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UNIVERSITY OF ALBERTA

CELL-MATRIX INTERACTIONS OF THE MESODERM IN THE GASTRULATING CHICK EMBRYO

BY

AMANDA J. BROWN ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE IN CELL BIOLOGY

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING 1991

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CELL-MATRIX INTERACTIONS OF THE MESODERM
IN THE GASTRULATING CHICK EMBRYO

SUBMITTED BY **AMANDA J. BROWN**

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **MASTER OF SCIENCE**

IN CELL BIOLOGY

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Date: 14th December, 1990

To my parents,

Sandra and Derrick,

for their silver wedding anniversary.

ABSTRACT

Gastrulating stage 3-4 chick embryos were set up in New cultures, and microinjected with either the fibronectin cell-binding peptide, RGDS; the laminin cell-binding peptide, YIGSR; the control peptide, GRGESP; anti-fibronectin; anti-laminin antibodies, or Pannett and Compton's saline. Embryos were then left in an incubator at 37°C for 6 hours. Light microscopy revealed that embryos treated with RGDS or anti-fibronectin antibodies showed a significant decrease in the number of mesoderm cells migrating on the basement membrane of the ectoderm. Observations using scanning electron microscopy, and a shape factor analysis program revealed that, in general, embryos treated with RGDS or anti-fibronectin antibodies also displayed mesoderm cells that were rounder in morphology, in comparison to other treatments.

In addition to the *in vivo* experiments, mesoderm cells from stage 5 embryos were removed and cultured on either fibronectin-coated, laminin-coated, or untreated glass coverslips, and exposed to medium containing the above listed peptides and antibodies, in addition to anti-fibronectin receptor antibodies, for a period of 24 hours at 37°C. It was found that RGDS, anti-fibronectin, and anti-fibronectin receptor antibodies prevented the mesoderm cells from growing on fibronectin-coated coverslips. YIGSR, and anti-laminin antibodies inhibited the growth of the cells on laminin. Endodermal tissue treated with anti-fibronectin receptor antibodies, however, continued to grow and maintain its usual epithelial morphology.

Results from these experiments lead to the conclusion that fibronectin, and to a lesser extent, laminin, both of which are present in the gastrulating chick embryo, may be involved in the migration of the mesoderm cells, using the basement membrane as a substratum. It is apparent from these observations that the cell-binding tetrapeptide, RGDS, present in fibronectin, is important in mediating the attachment of the mesoderm cells to fibronectin, since addition of the peptide *in vivo*, causes distinct morphological changes in the cells. However, RGDS appears not to be the only site responsible for cell attachment. It is more than likely that a number of factors are contributing to the continuing migration of the mesoderm cells during gastrulation.

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INTRODUCTION

How can a two-dimensional genetic code specify the development of the complex three-dimensional shape of animals? This is a central question that embryologists have been asking for many years. Although there are numerous aspects of development that are still unknown, the basic concepts have been identified. It is generally believed that the development of multicellular organisms can be divided into three phases. These are differentiation, pattern formation and morphogenesis.

Differentiation involves the permanent, structural and functional specialization of individual cells from one of a number of common cell types, which possess the capability to develop in several different ways. For example, the mesenchyme cells of the embryonic chick limb bud may become muscle or cartilage cells. Such differentiation is largely an intracellular process, and involves both the biochemical and cytological characterization of the cells. In skeletal muscle, for example, the specific proteins, actin and myosin are synthesized, and these are arranged specifically within the cell giving it the typical striated appearance.

Pattern formation can be defined as the spatial organisation of differentiated cells. It is the consequence of these differentiated cells, and their correct spatial and proportional relationships to each other, that ensures the continuing development of the embryo in a precisely defined manner.

The third process involved in embryogenesis is morphogenesis. This is a mechanical process, whereby the form of the organism, and the arrangement of its tissues are generated. This is achieved by the co-ordinated movement of cells, either individually or as groups. One of the most dramatic examples in most organisms is gastrulation.

Although all three processes are important in the development of the embryo, without morphogenesis and the movement of cells, there would be no interaction between different cell types or any of the inductive processes that occur between different tissues, and are so important to the continuing development of the embryo. Therefore, this subject shall be considered in greater depth.

Considering morphogenesis, two questions come to mind. Firstly, how do cells move, and secondly, are their movements coordinated? In most cases, it is impossible to make direct observations of cell movements in embryos, because the majority of embryos are not transparent. For this reason, most of the work relating to these questions has been performed on cells in artificial situations, such as tissue culture.

It appears that certain cells may be responding to intrinsic factors and are actually programmed for their directionality (Bellairs, 1982). This may be illustrated by the use of HNK-1 monoclonal antibody (Abo & Balch, 1981), which binds to a sulphated sugar moiety that is expressed on several cell-surface glycoproteins involved in adhesive interactions. This monoclonal antibody is capable of recognising cells in the chick embryo that will eventually enter the primitive streak (Canning & Stern, 1988). Other antigens and lectins have also been found to associate with certain cells at certain stages of development, indicating that possibly the fate of certain cells is determined from the very early stages of development.

Observation of migrating cells has shown that they can extend two types of cellular processes, namely filopodia and lamellipodia (for review see Dunn, 1980). Filopodia are seen as long, fine cellular processes which extend and adhere to the substratum. As the filopodia shorten, so the cell is drawn closer to the point of attachment. It is believed that microtubules are involved in the extension of the filopodium, and microfilaments in their contraction (Abercrombie, 1980; Izzard & Lochner, 1980; Rinnerthaler *et al.*, 1988). Lamellipodia, however, are flatter, and more wide-spread than filopodia, and possess ruffles at their leading edge. It was hypothesized by Ingram (1969), that the leading lamellipodium attaches to the substratum a short distance in front of the cell, and then contracts, pulling the cell forward. By repeating this sequence, the cell could continue its forward movement.

In addition to the influence by possible intrinsic factors, cells are also exposed to a number of extrinsic, or environmental factors. These include chemotaxis, haptotaxis, galvanotaxis, contact guidance, contact inhibition of locomotion, and cell contacts.

Chemotaxis can be described as the localised secretion of a chemical that leads to the directional guidance of cell movement. One of the best examples of chemotaxis in development can be seen in *Dictyostelium discoideum*, the slime mould. During its life cycle, the slime mould undergoes a series of morphogenetic changes leading to the formation of a fruiting body. The initial part of the cycle is a feeding phase, during which time the amoeboid cells consume bacteria and other micro-organisms. Once the food supply is exhausted, the second stage of the cycle begins, and the cells come together to form loose clusters. It is from these aggregation sites that chemotactic signals emanate, causing the continuing accumulation of cells at these sites. It was shown that 3'5'-cyclicAMP was, in part at least, involved in the chemotactic process (Konijn *et al.*, 1967). The cells gradually pile up to form a mound, and from this stage onwards, behave as an organism in its own right. Eventually, the cell mass rises vertically, and a fruiting body is formed at the tip of the stalk.

Chemotaxis has been shown to be an important process in the development of *Dictyostelium discoideum*. To date, however, it has not been shown definitely, to be a contributing factor in the movement of cells *in vivo* in early vertebrate development, although it has been implied (Greenberg *et al.*, 1981; Albini *et al.*, 1987).

Haptotaxis is the accumulation of cells in a particular location in response to an adhesive gradient in the substratum (Carter, 1967). Although this effect has been demonstrated a number of times *in vitro*, one example being the response of melanoma cells to gradients of fibronectin and laminin (McCarthy & Furcht, 1984), there appears to be no firm evidence for this effect *in vivo*, during early vertebrate development. Fibronectin has been shown to be present in the migratory pathways of many migrating cells, and, although no adhesive gradients have been demonstrated, it may provide a mechanism for cell movement, by providing a substrate on which to migrate (Newgreen & Thiery, 1980; Duband & Thiery, 1982; Boucaut *et al.*, 1985).

Galvanotaxis is the influence, or directional migration of cells in response to an electrical field (Robinson, 1985). Although such a phenomenon has been demonstrated by a variety of cells in culture, there is no direct evidence to show that it exists in the developing embryo *in vivo*. There is a possibility, however, that embryos may, in fact, generate electrical fields, by the outward leaking of ions that are actively being pumped across epithelia (see Jaffe & Stern, 1979).

Contact guidance is the process whereby cell shape, orientation and the direction of movement, are influenced by topographic heterogeneities in the substratum, such as grooves or fibres (Weiss, 1955; Dunn, 1982). This phenomenon has been demonstrated *in vitro*, one example being the work performed by Dunn & Ebendal (1978), where it was shown that scratches on a glass petri dish, or orientated collagen fibrils, could cause cells to align along these discontinuities. Examples of contact guidance occurring *in vivo* have also been demonstrated (see Trinkaus, 1982), but it is believed that it must occur in conjunction with other influences, such as contact inhibition of locomotion, or population pressure, to maintain a forward movement of cells. In the gastrulating chick embryo, it was observed that at the rostral boundary between the area pellucida and the area opaca, there was a band of parallel, fibronectin-rich fibres (Critchley *et al.*, 1979; Wakely & England, 1979). It was at first believed that these fibres were perhaps a contact guidance system, but later work by Andries *et al.*, (1985) showed that these fibres were not a favourable substratum for cell spreading. There appears not to be, therefore, any direct evidence to date, that suggests that the migrating mesoderm cells in the chick embryo use any form of contact guidance.

Contact inhibition of locomotion is the cessation of locomotion after two cells contact, and the subsequent redirection of movement away from the point of contact (for review, see Heaysman, 1978). Although such a phenomenon has been demonstrated *in vitro* (Gooday & Thorogood, 1985; Erickson, 1988), it is difficult to demonstrate contact inhibition of locomotion *in vivo*, since cells cannot be filmed at a high enough resolution *in vivo* in the early embryo. The possibility of contact inhibition of locomotion influencing cell movement during morphogenesis should not be ruled out, however.

The final category to be considered here, are cell contacts. These are involved in the adhesion of cells to their substrata, and to each other, and are, therefore, important in influencing cell movements that occur during morphogenesis. Intercellular contacts can generally be grouped into four categories 1) the tight junction (zonula occludens) 2) the gap junction 3) the adhaerens-type junction (intermediate junction) and 4) the desmosome (macula adhaerens).

The tight junction is the apical-most contact between cells, and forms a belt around each epithelial cell, causing a barrier, or seal against the passage of molecules between the cells. When seen in freeze-fracture, the tight junction appears as a series of branching and anastomosing ridges and grooves apparent on each surface of the plasma membrane. In addition to their function as a barrier, they are also regions of firm intercellular adhesion, and also prevent lipids in the apical region of the outer plasma membrane from diffusing to the basolateral domains (Dragsten *et al.*, 1981). Two potential markers for tight junctions have recently been discovered. These are the proteins ZO-1 (Stevenson *et al.*, 1986; Anderson *et al.*, 1988), and cingulin (Citi *et al.*, 1988), both of which appear to be localised a slight distance away from the tight junction on the cytoplasmic side (for review see Cereijido *et al.*, 1989).

Gap junctions are the most common of the specialized junctions, and can be found in both mesenchymal and epithelial cells. They appear early in embryogenesis, and because their distribution and permeability seem to change at particular times during development, it has been proposed that they may have a potential role in differentiation. Gap junctions consist of a collection of hexagonal subunits, called connexons (for review see Beyer *et al.*, 1990). Connexons in one cell associate with similar subunits in another cell, and form a continuous aqueous path between the cell cytoplasms. This may lead to the sharing of regulatory ions *eg.* calcium, by coupled cells and could coordinate morphogenetic activities. This coupling has been demonstrated experimentally, by measuring the flow of electrical current directly between cells, or by observing dye transfer from one cell to another. This demonstration, however, has not directly shown that gap junctions in fact mediate this process. Bellairs *et al.*, (1975) showed that gap junctions are present in the chick even before the development of the two-layered embryo. Further studies on the gap junction in chick development, however, have not been carried out, and so their function is still unknown.

The adhaerens junctions are found encircling the apical regions of columnar epithelia, just basal of the tight junction complex. They appear to be associated with actin filaments (Green *et al.*, 1987). This could be of great significance for embryonic epithelia, since the actin filaments associated with these junctions could cause a contraction of the apical end of the cell, and may be responsible for the process of tissue folding. It has been shown that early embryonic cells in culture are capable of rapidly forming adhaerens-type junctions, and it is though that this may be correlated with the cohesiveness of the tissue *in situ* (Sanders & Prasad, 1981). In addition to adhaerens junctions between cells, they are also present between cells and their substrata, and appear as half-junctions. These focal contacts represent points of firm attachment, and with actin filaments (stress fibres) terminating at this point, are thought to be vitally important for cell locomotion *in vitro*.

The final junction to be considered here, is the desmosome (for review see Gorbsky, 1986). It occupies a round or oval space on the lateral cell surface, and the apposed plasma membranes are usually separated by a 25-35nm intercellular space containing electron dense material. Associated with the desmosome (on the cytoplasmic side) are intermediate filaments (tonofilaments). Although desmosomes are present in the epithelia of adult and late embryonic tissues, they are not as abundant in early embryonic tissue, indicating perhaps, that their importance in morphogenesis during the early stages, is not as great as other types of junctions. The plaques of the desmosome contain a number of proteins, including the desmoplakins, which extend from the plasma membrane through the cytoplasmic plaque, and the desmogleins, which appear to be important in adhesion, since they extend from the midline of the intercellular space across the plasma membrane, and into the cytoplasmic plaque.

In addition to adhesive complexes, such as the aforementioned junctions, being involved in cell-cell and cell-substratum attachment, adhesion is also maintained through independent molecular sites of recognition, namely cell adhesion molecules

(CAMs). It is believed that CAMs may take a key role in the first steps of recognition and adhesion between cells, and may also participate in the formation of adhering junctions.

An important property of cells relating to their morphogenetic capacity is their ability to be able to distinguish between identical or different cell types, and to adhere, preferentially, to their own cell type, when mixed with others. Such selectivity in cell-cell adhesion would be expected to have a key role in the organization of tissues.

Takeichi (1977) divided the CAMs into two systems - those that were calcium-dependent (cadherins), and those that were calcium-independent. The two best studied CAMs are N-CAM and L-CAM, which were originally isolated from neural and liver cell membranes respectively (see Edelman, 1983). Binding of N-CAM has been found to be homophilic *ie.* identical CAMs expressed on different cells can bind one to the other. Two other CAMs were later isolated, and these were referred to as A-CAM (Volk & Geiger, 1984), and Ng-CAM (Grumet & Edelman, 1984). Although it was initially found in cardiac muscle, A-CAM is now thought to be a membrane-bound glycoprotein, that is almost exclusively found in adhering junctions of some epithelial cells. Ng-CAM was originally identified in an *in vitro* assay, in which glial cells bound to brain cell membrane vesicles.

Both N-CAM, and L-CAM have been studied extensively during the development of the chick embryo (Thiery *et al.*,1982; Edelman *et al.*,1983). Before gastrulation both CAMs are expressed in low levels on the epiblast and hypoblast. During gastrulation, however, the epiblast loses expression of both N-CAM and L-CAM as cells begin to ingress, although L-CAM later reappears on the ectoderm and endoderm, but not the mesoderm. Ng-CAM, on the other hand, is not detectable in the chick embryo until three days after the onset of development, in cells of the ventral neural tube (Thiery *et al.*, 1985). It appears throughout development that changes in CAM expression occur during epithelial-mesenchymal transformations, and in association with sites of inductive interactions. This has led to the belief that CAMs play an important role in bringing both cells and tissues together, and are, therefore, a major contributing factor in the morphogenetic process (for review see Takeichi, 1988).

It is apparent from this discussion, that there are many mechanisms involved in, not only morphogenesis, but also in embryogenesis as a whole. Many of these mechanisms have been shown to be present in cells cultured *in vitro*, but to demonstrate directly, that they are contributing factors *in vivo*, has proven to be more arduous. One such example can be found during the early stages of chick development, when cells are migrating to positions within the embryo that will eventually determine their fate. Since the observations by Trelstad *et al.*, (1967) concerning the migration of the mesoderm cells, and their utilization of the basement membrane, there is now more information on individual components of the basement membrane, and the way in which they may affect and influence cells which use them as substrates.

The aim of this project was to investigate the relationship between cell movement observed in the migrating mesoderm during gastrulation, and the attachment of these cells to various extracellular molecules of the basement membrane. Although many of the aforementioned systems occurring during morphogenesis may be, in part at least, involved in the migration of the mesoderm, this project concentrates on the aspect of cell-substratum adhesion, in particular the involvement of the two most characterized glycoproteins of the basement membrane; fibronectin and laminin.

Research involving the use of synthetic peptides, particularly those involved in normal and transformed cell attachment to fibronectin, is quite widespread, and not confined to mammalian systems (Naidet *et al.*, 1987). The present investigation has taken advantage of the synthetic peptides now available, in particular, the cell-binding regions of both fibronectin and laminin. By microinjecting the synthetic peptides into the developing embryo, it has been possible to interfere with the normal migration processes, in the hope that it will lead to a greater understanding of the relationship between cells and their substrata, particularly in the developing chick embryo.

LITERATURE REVIEW

GASTRULATION IN THE CHICK EMBRYO (*Gallus domesticus*)

Lewis Wolpert is once supposed to have said, 'it is not birth, marriage or death, but gastrulation which is truly the most important time in your life' (quoted by Slack, 1983).

In avian development, gastrulation is generally thought to comprise of a complex series of events, whereby cells from the epiblast layer invaginate through the primitive streak, giving rise to endoblast and mesoderm cells. This process involves a transformation from epithelial to mesenchymal cell morphology, and converts the two-layered blastoderm into a three-layered embryo. In addition to these changes, gastrulation is also causal in changing the embryo from its initial radial symmetry, to a bilateral symmetry (Stern & Canning, 1988).

By the time the egg is laid, the blastoderm is approximately 2mm in diameter, and can be seen to consist of a disc of cells, developing between the yolk and the vitelline membrane. The blastoderm consists of two regions; the area pellucida, which appears transparent, and is the region in which the primitive streak develops, and peripheral to that, the area opaca, the edges of which are attached to the vitelline membrane, maintaining the embryo's position. The embryo itself consists of two layers. The upper layer, or epiblast, is a pseudostratified epithelium, determined by the fact that all of its cells rest on the basement membrane, although not all of them reach the free surface. The epiblast lies directly beneath the vitelline membrane, and is continuous over both the area pellucida and opaca. The lower layer, or hypoblast, is found lining the subgerminal cavity between the yolk and the epiblast, and is initially only present at the caudal end (Low, 1967).

At this point, the blastoderm has a definite polarity (Eyal-Giladi & Kochav, 1976), possessing, dorso-ventral and rostro-caudal axes. The caudal region is easily distinguishable, since the area opaca is slightly broader towards at the rostral end of the embryo (Bellairs, 1982). It is at the caudal end of the embryo that the primitive streak develops, and extends rostrally, as a direct result of the moving epiblast.

It is thought that the invagination of the epiblast is not a random process, but is, to a certain extent, preprogrammed (Bellairs, 1982). Using scanning electron microscopy, it was shown that epiblast cells destined to invaginate through the primitive streak, displayed a characteristic blebbing on both their dorsal surface (Bancroft & Bellairs, 1974) and ventral surfaces (Vakaet, 1984). As the cells reach the point of invagination, they become "flask-shaped", as they extend ventrally through the primitive streak, attached to adjacent cells by filopodia and lamellipodia (Ebendal, 1976; England & Wakely, 1977; Solursh & Revel, 1978). Cell shortening then occurs, which pulls the cell into the interior of the embryo. Cytoplasmic microtubules have been shown to be abundant in streak cells, lying parallel to the long axis of the cell (Sanders & Zalik, 1970). This would suggest that they have a function in the altering shape of the cells, and contributing to the passage of the mesoderm through the streak.

Once the cells have ingressed, they differentiate into one of two cell types. They may become definitive endoblast, which integrates into the lower layer, and gradually displaces the hypoblast distally (Stern & Ireland, 1981). These cells will eventually become embryonic endoderm (Fontaine & Le Douarin, 1977; Sanders *et al.*, 1978). By stage 4 (Hamburger & Hamilton, 1951) the hypoblast is confined to the rostral margin where the area pellucida and area opaca meet, and is referred to as the germinal crescent. This will form the extraembryonic endoderm (Fontaine & Le Douarin, 1977; Sanders *et al.*, 1978). Alternatively, the invaginated cells may become mesoderm cells, and migrate laterally away from the streak, to eventually differentiate into skin, muscle and skeletal components. Observation of these cells shows that they come into contact with the basement membrane present underlying the ectoderm, in addition to the dorsal surface of the endoderm, and also with each other (Trelstad *et al.*. 1967). Mesoderm cells within the streak region have been shown to display elevated levels of activity of hyaluronidase (Stern, 1984). Since the basement membrane of the overlying ectoderm consists, in part, of hyaluronate, (Sanders, 1979; Solursh *et al.*, 1979) it may be possible that this high level of enzyme activity could be responsible for the degradation of the basement membrane, by the mesoderm, to encourage more cells to ingress (Stern, 1984).

It is believed that the primary purpose of the hypoblast, is to exert an inductive influence on the overlying ectoderm, thereby triggering certain cells in the ectoderm to change their developmental fate, and become primitive streak cells (Waddington, 1930; Azar & Eyal-Giladi, 1979). Experiments have been performed, where the ectoderm was separated from the hypoblast, and a millipore filter was placed between the two tissue layers (Eyal-Giladi & Wolk, 1970). It was found that when the hypoblast was present on the opposing side of the filter, there was an increased incidence of the development of a primitive streak in the ectoderm on the other side. By rotating the hypoblast either 90 or 180 degrees, in cultured embryos, the primitive streak in the ectoderm could be encouraged to form in parallel with the orientation of the hypoblast, indicating a definite role for the hypoblast in primitive streak induction (Azar & Eyal-Giladi, 1981).

As the cells continue to invaginate, the primitive streak extends rostrally, mainly due to the movement of more rostrally situated ectoderm toward the midline. This continuing invagination creates a groove, which is apparent on the surface of the blastoderm. The groove terminates rostrally at a point referred to as the primitive pit, although the primitive streak extends slightly anterior from this point, and terminates in a clump of cells known as Hensen's node. It has been postulated that rod-like appearance of the primitive streak is created by two simultaneous forces. The tension generated by the expansion of the blastoderm on the vitelline membrane creates one force, whilst the change in shape, arrangement, and proliferation of the cells in the ectoderm region of the primitive streak creates a second (Bellairs, 1986). The combined forces result in the characteristic appearance of the primitive streak.

Factors influencing the migration of the mesoderm cells laterally away from the primitive streak

It is well known that fibronectin promotes cell migration, and plays an important role in the guidance of cells along their migratory pathways (Ali & Hynes, 1978; Rovasio *et al.*, 1983). It is possible that fibronectin could be involved in the continued migration of the mesoderm cells away from the primitive streak. An example of fibronectin involvement in the migration of cells was demonstrated by Newgreen and Thiery (1980) who showed that avian trunk neural crest cells and primordial germ cells were unable to synthesize fibronectin *in vitro*, but they were capable of synthesizing fibronectin *in vivo*, when they were migrating next to their pathways. Newgreen and Thiery postulated that the lack of fibronectin synthesis by migrating cells may increase their sensitivity to exogenous fibronectin produced by pathway-forming cells, therefore providing cues to guide migration. Tracks of exogenous fibronectin by pathway cells could also specify the direction of migration for the cells. This can be seen during the development of the avian heart, when precardiac cells, and endocardial cushion cells appear to migrate along gradients of fibronectin (Linask & Lash, 1986), a condition known as haptotaxis, where cells move from a less adherent to a more adherent surface (Carter, 1967). Ffrench-Constant and Hynes (1988), however, disagreed with Newgreen and Thiery's hypothesis, that the lack of fibronectin by migrating cells would make them more sensitive to exogenous fibronectin. They discovered that mesoderm cells of the area vasculosa, the region in which blood vessels and erythrocytes develop, and endocardial cushion cells both contain the same mRNA for fibronectin, as their pathway-forming cells. This led them to believe that fibronectin may be important for cell movement, but not necessarily for cell guidance. Another mechanism that could promote the migration of the mesoderm cells away from the primitive streak, is contact inhibition of locomotion. Contact inhibition was defined as 'the stopping of the continued locomotion of a cell in the direction which has produced a collision with another cell, or alternatively, the prohibition, when contact between cells has occurred, of continued movement such as would carry one cell across the surface of another' (Heaysman, 1978). *In vitro*, this phenomenon leads to an inhibition of the ruffling membrane at the leading edge of the cell, followed by the retraction from the point of contact and a change in the direction of movement. This leads to a radial movement of cells, away from the tissue explant. Contact inhibition may also be a contributing factor in the migration of cells *in vivo*. In addition to the finding by Rovasio *et al.*, (1983) that neural crest cell migrated on tracks of exogenous fibronectin, they also determined that a high cell density was also needed. Such may be the case in the migration of the mesoderm cells in the developing chick embryo.

Spatial patterning of antigens during gastrulation

Throughout the extensive experimental studies that have been performed on chick embryos, it has become increasingly apparent that there is often a correlation between the morphogenetic events that take place during embryogenesis, and the distinct temporal and spatial patterning of certain antigens. One such example, is the monoclonal antibody, HNK-1 (Abo & Balch, 1981), which binds to a complex sulphated sugar moiety, which is expressed on several cell-surface glycoproteins involved in adhesive interactions. It is capable of recognising cells, in the chick embryo, that will eventually enter the primitive streak (Canning & Stern, 1988). When chick blastoderms are treated with HNK-1, it can be seen to be randomly distributed within the ectoderm, and will only be associated with those cells that invaginate to form mesoderm and endoderm (Stern & Canning, 1990). If HNK-1 positive cells are ablated, the remaining ectoderm cannot form mesodermal structures *ie.* they are unable to alter their phenotype. After streak formation, the ectoderm displays a rostro-caudal gradient of HNK-1 epitope expression (Canning & Stern, 1988). Beyond this point in gastrulation, the epitope gradually disappears, until the development of the notochord, which is the next tissue to display HNK-1 binding. Since the HNK-1 epitope is associated with ectoderm cells entering the primitive streak, and the notochord, both of which are undergoing inductive interactions, it has been speculated that HNK-1, or related sugars may be a general feature of inductive processes such as these (Canning & Stern, 1988). *In vitro* studies with neuronal cells, have indicated that the epitope is of functional importance in both cell-substrate and cell-cell interactions (Kunemund *et al.*, 1988).

Another example of an antigen that is cell-specific, is FC10.2 (Loveless *et al.*, 1990). Oligosaccharides, present at the cell surface, are composed of two main glycoprotein backbone structures consisting of repeating disaccharide units of galactose and N-acetylglucosamine. Different linkages of the non-reducing terminal galactose residue gives rise to two types of oligosaccharide chains. Type 1 is composed of galactose ß1-3 linked units, whilst type 2 consists of galactose ß1-4 linked units (Watkins, 1980). The antigen, FC10.2, recognises the presence of the type 1 backbone structure. In this particular study, FC10.2, and its sialated form, S-FC10.2, were detected in chick embryos from stage 1 (unincubated egg; Hamburger & Hamilton, 1951), to stage 17 (30 pairs of somites; Hamburger & Hamilton, 1951). Cells leaving the ectoderm, and ingressing into the primitive streak did not express FC10.2 or its sialated form. However, as the cells began to migrate laterally away from the streak, and form paraxial mesoderm, they expressed, predominantly, S-FC10.2. Throughout all the stages studied, the antigen appeared to be a clear marker for the primordial germ cells. It was speculated, that antigens such as these, may be involved in the binding with adhesion molecules. Thorpe *et al.*, (1988), also demonstrated distinctive changes in the distribution of antigens associated with another series of cell-surface carbohydrates, the poly-N-acetyllactosamines. These were found to have distinct rostro-caudal, mediolateral, and dorsoventral patterns of expression in both the epithelia, and the extracellular matrix, and, like the antigens previously mentioned, showed striking changes in their distribution throughout the developmental stages, indicating a possible functional role for these structures during early embryogenesis.

In addition to the carbohydrate markers already mentioned, an endogenous galactose-binding lectin has been demonstrated to be present in the chick embryo (Zalik *et al.*, 1987). In the area opaca, the intracellular lectin is associated with the ectoderm, but is also present in large concentrations in the endoderm, and the primordial germ cells. Within the area pellucida, however, the lectin is not present in the ectoderm, but is expressed as the cells migrate inwards towards the streak, and emerge laterally, to form the endoderm.

Intermediate filaments are cell-type specific proteins that are expressed by most cells. A study of the early stages of chick embryogenesis, by Page in 1989, revealed that predominantly two types of intermediate filament were present. Vimentin, a mesenchyme-specific protein, was found to be associated with cells destined to ingress through the primitive streak, to form mesoderm and definitive endoderm. Cytokeratin, an epithelial-specific protein, was found, initially, to be restricted to the ectoderm overlying the area opaca and the marginal zone. It appears therefore, that cytokeratin is associated with cells that are destined to remain in the ectoderm, whilst vimentin is associated with the expression of reduced cell contact (Connell & Rheinwald, 1983). In agreement with this, it has also been found that under low cell density conditions, epithelial cells can switch their normal expression of cytokeratin, to predominantly, vimentin. Under high cell density conditions, this situation is reversed (Ben Ze'ev, 1984). Although the distribution of these two proteins appears to be very well defined, their precise function and the significance of their cell-type specificity is ambivalent.

(For reviews on gastrulation, see Nicolet, 1971; Sanders, 1986; Stern & Canning, 1988).

THE EXTRACELLULAR MATRIX AND BASEMENT MEMBRANE OF THE GASTRULATING CHICK EMBRYO.

The extracellular matrix (ECM) of early embryos consists of a complex network of material, that can be found underlying epithelia, surrounding mesenchymal cells, and providing a substratum for cell attachment. Generally speaking, the ECM can be classified into two principal types, the basement membrane matrix, between epithelial cells and connective tissue, and interstitial matrix, between connective tissue cells (see Hay, 1981). Both types of matrix can be divided into three main classes of molecules, including the glycosaminoglycans or

GAG's (the major constituent of proteoglycans), the collagens, and the non-collagenous glycoproteins. The ECM, including the basement membrane, is thought to exert both mechanical and chemical influences on the shape and biochemistry of the cells, via the plasma membrane (Hay, 1982).

Glycosaminoglycans are high molecular weight unbranched polysaccharide chains, consisting of repeating dimers of an amino-sugar (N-acetylglucosamine or N-acetylgalactosamine) alternating with a uronic acid residue (D-glucuronic acid or N-acetyl-D-glucosamine repeating disaccharide). One of the major GAG's present during gastrulation, is hyaluronate (Solursh, 1976; Fisher & Solursh, 1977; Vanroelen *et al*, 1980), which is not sulphated, and accounts for approximately 84% of the GAG's present at this time. Since hyaluronate can be extensively hydrated, it is thought that its function is to enlarge extracellular spaces, promoting the detachment of the ingressing cells, and allowing the migration of the mesoderm. It is probable that the fluid which is absorbed by the hyaluronate, is derived from the egg white. It has been demonstrated *in vitro*, that the accumulation of fluid in the subgerminal cavity is due to the blastoderm actively absorbing fluid from the albumin on its ectodermal surface, and secreting it from its endodermal surface (New, 1956). In the rat embryo, it has been demonstrated that a glycoprotein, termed hyaluronectin, is capable of binding mesenchymal cells to hyaluronate (Delpech & Delpech, 1984). If GAG-degrading enzymes are injected into the gastrulating chick embryo, the mesoderm cells become compacted, and they withdraw any cellular processes (Fisher

& Solursh, 1977; Van Hoof *et al*, 1986). Proteoglycans consist of unbranched GAG's, including chondroitin 6-sulphate, chondroitin 4-sulphate, heparan-sulphate, dermatan sulphate and keratin sulphate. They are linked, often in a mixture, via their serine residues, to a core protein (Heinegard & Sommarin, 1987). Basement membranes are rich in heparan sulphate proteoglycan.

The collagen molecule is a triple-stranded helical structure, composed of three α-polypeptide chains. There are approximately twenty α chains known to date, which are expressed in different combinations in different tissues. The best defined collagens, are types I, II, III, and IV. Type I collagen is the most common, and, along with types II and III, make up the fibrillar collagens, which are thin polymers that aggregate into large bundles called collagen fibres. Type IV collagen, however, which is found exclusively in basement membranes, does not fall into this category, but forms a sheet-like network that constitutes a major proportion of the basement membrane. During avian gastrulation, it appears that there is a scarcity of fibrillar elements, however, labelling of primitive streak stage chick embryos with ^{3}H-proline has revealed that, in both the area pellucida, and area opaca, interstitial type I and type III collagens are present, in addition to basal lamina type IV collagen (Manasek, 1975; Hay, 1981). As development proceeds, so the distribution of the various collagen types becomes more diverse.

The third class of molecules present in the ECM, are the non-collagenous proteins, which appear to be involved in interactions between other matrix constituents, and the cell surface. Two of the better characterized members within this class are fibronectin, and laminin. A detailed discussion of these molecules can be found later on in this review.

The second class of matrix, as previously mentioned, is the basement membrane. It is an insoluble, distensible structure, which is impermeable to large proteins (Kefalides, 1979; Martinez-Hernandez & Amenta, 1983), and is usually found at the basal surface of epithelial cells. In addition to their function as a selective barrier, that is, a molecular filter of macromolecules, in regions such as the glomerulus of the kidney, and a barrier to cell penetration, basement membranes also provide physical support, and a substratum for cell attachment.

In general, the basement membrane consists of three layers (Laurie & Leblond, 1985). Directly in contact with the plasma membrane of the cell, is the lamina lucida, which is between 50 and 80nm thick, and is transversed by fibrils. Overlying the lamina lucida, is the lamina densa, which has approximately the same thickness. The third region, which is not as well defined as the other two layers, is the lamina fibroreticularis, which blends into the general extracellular matrix of the connective tissue. As a rule, most basement membranes consist of a common core of components, including type IV collagen, laminin, entactin and heparan sulphate proteoglycan. Depending on the function of the basement membrane, it may also contain fibronectin and/or type V collagen. The main structural component of basement membranes, however, is the triple-helical type IV collagen molecule, which forms a tetramer, stabilized by disulphide bridges and non-reducible cross-links (see Kefalides *et al.*, 1979; Timpl & Dziadek, 1986). It is derived from three polypeptide chains [$\alpha1(IV)_2$ $\alpha2(IV)$], measures 400nm in length, and possesses a distinct globular domain at its carboxyl terminus (Yurchenco & Schittny, 1990). Type IV collagen forms a three-dimensional network, and provides a scaffold for the attachment of other molecules. In basement membranes lacking substantial amounts of collagen, for example, in embryogenesis (Timpl, 1989), laminin may provide the only polymer framework (for more detail, see section on laminin).

Entactin (Carlin *et al.*, 1981), and nidogen (Timpl *et al.*, 1983) are believed to be different names for the same macromolecule. It is approximately 17nm long, shaped like a dumbbell, and has been found to exist in tight association with laminin in a 1:1 complex (Paulsson *et al.*, 1987). Since nidogen is capable of binding to both laminin and type IV collagen (Aumailley *et al.*, 1989), it appears that it may act as a bridge between the two major basement membrane proteins.

Heparan sulphate proteoglycans are a class of heterogeneous macromolecules characterized by a protein core covalently bound to heparan sulphate chains. The interaction of sulphated glycosaminoglycans with other basement membrane components, may allow the coordinated regulation of basement membrane structure and function.

In 1967, Trelstad *et al.* postulated that the mesoderm cells, moving laterally away from the primitive streak, used the developing basement membrane as a substrate on which to migrate. It has also been shown that the ECM and the basement membrane, play a crucial role in epithelial-mesenchymal interactions. One such example is found during the morphogenesis of the mouse embryo submandibular salivary gland (Bernfield *et al*, 1984). The development of this branching structure relies on the interaction of the mesenchyme and epithelium, which, in turn, regulates the accumulation and degradation of the basement membrane. This leads to a modification of the epithelial behaviour, and initiates the branching morphogenesis. Changes in ECM composition appear to coincide directly with developmental changes in a number of interacting systems. The precise role of the various basement membrane components, has not, however, been fully established in any system.

It appears that, from the unincubated stage onwards, staining of chick embryos with tannic acid, reveals an organised basement membrane, complete with lamina lucida, and lamina densa (Sanders, 1979). In the region of the primitive streak, however, observations have shown that there is a disruption of the basement membrane (Low, 1967; Duband & Thiery, 1982), and a concomitant reduction in the amount of fibronectin and laminin (Mitrani, 1982; Bortier, *et al.*, 1989). This may be an important factor in precipitating invagination of cells. In the mouse embryo, laminin is present from an early stage, and is found exclusively in the basement membrane (Leivo *et al.*, 1980). In the quail blastoderm, laminin can be found on the ventral surface of the future epithelial cells, before ovulation has occurred, indicating that these cells produce the laminin of their basement membrane. With immunofluorescent staining, laminin appears as a continuous linear band throughout the area pellucida (Mitrani, 1982). Fibronectin, not unexpectedly, was also found in the basement membrane, with similar staining patterns to laminin (Mitrani & Farberov, 1982), but less specifically localized, and in greater abundance than laminin. The intensity of staining did not differ much between the time of laying, and gastrulation (Critchley *et al.*, 1979; Sanders, 1982). During migration of the mesoderm, fibronectin was detected in association with all parts of the ectoderm, particularly in the basement membrane, although never actually in the mesoderm itself (Critchley *et al.*, 1979; Duband & Thiery, 1982). This would suggest that the mesoderm cells do not deposit fibronectin during their migration, but instead, utilize the fibronectin-rich basement membrane present on the ectoderm as a substratum. Recent work on the molecular biology of fibronectin has demonstrated that it is a heterogenous molecule, and that the heterogeneity is generated, *in vivo*, by an alternative splicing of the fibronectin gene transcript (Hynes, 1985; Kornblihtt *et al.*, 1985; Norton & Hynes, 1987). Such splicing alterations may, therefore, produce various forms of fibronectin, with differing adhesive properties, which may play different roles in the promotion and guidance of cell migration. In this way, the distribution of alternative types of exogenous fibronectin within the extracellular matrix, may represent a mechanism for pathway specification (Ffrench-Constant & Hynes, 1988). In addition, differential glycosylation of fibronectin in various embryonic regions or phases of development, may possibly determine the availability of binding sites, and thereby influence the association of fibronectin with components of the extracellular matrix and with the cell surface (Jones *et al.*, 1986).

At the rostral boundary of the area pellucida with the area opaca, a band of parallel fibronectin-rich fibres was observed, that extended caudally, and terminated at the border, in line with Hensen's node (Critchley *et al.*, 1979; Wakely & England, 1979). Andries *et al.*, (1985), demonstrated that cells present on this fibrous band, were of a low density, spherical in shape, and lacking lamellae, suggesting that this area was not a favourable substratum for cell spreading. They suggested, therefore, that the fibrillar band was not a contact guidance system, but may provide a barrier to stabilize the partition between the embryonic and extra-embryonic tissues in the rostral part of the blastoderm.

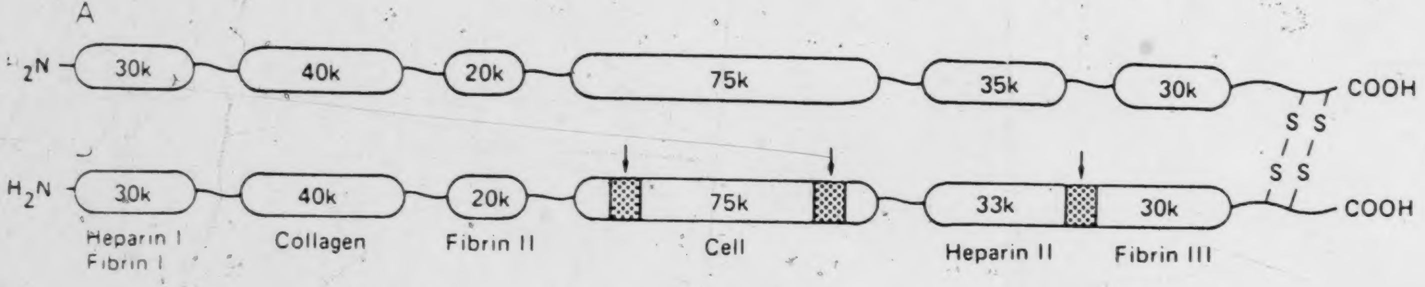
Although fibronectin is present in the basement membrane, the mesoderm cells have low levels of surface fibronectin, in comparison to the endoblast and hypoblast. This results in a low adhesiveness of the mesoderm cells, thereby facilitating migration (Sanders, 1980). Despite the fact that fibronectin has been shown to influence the movement of mesoderm cells *in vitro*, it is still unclear how fibronectin affects the mesoderm *in vivo*, if indeed it does.

FIBRONECTIN

It has already been emphasized that fibronectin plays a major role in cell attachment and migration in embryogenesis. In addition to this, it is also involved in opsonization (Saba *et al.*, 1978; Blumenstock *et al.*, 1978; Saba & Jaffe, 1980), wound healing (reviewed by Grinnell, 1982), and cancer (Vaheri & Ruoslahti, 1974; Yamada & Pastan, 1976; Olden & Yamada; 1977; Vaheri & Mosher, 1978). Since fibronectin has been implicated in a variety of cell contact processes, let us now consider it in a little more detail.

Fibronectin is a large glycoprotein consisting of two similar polypeptide chains, each with a molecular weight of approximately 250,000 kDa, and held together by two disulphide bonds, situated near the carboxyl termini. This class of multifunctional glycoproteins can be divided into two major groups: a plasma form, which is soluble, and found in body fluids, such as plasma, and cerebrospinal fluid, and a cellular form, which is synthesized by a wide variety of cells and organised into fibrillar structures, which represent an important constituent of ECM. Each subunit of fibronectin appears to be composed of discrete, globular domains (Alexander *et al.*, 1978), which are separated by short, flexible segments. The domains, which contain the binding sites for a variety of molecules, including collagen, fibrin, fibrinogen, heparin, DNA, actin, as well as a region which is capable of binding a variety of cell types, tend to be relatively resistant to proteases. The interdomain regions, however, can easily be cleaved (for primary structure of fibronectin see Figure 1).

Figure 1 - primary structure of the fibronectin molecule, showing the RGDS cell-binding site, and two other regions (arrows) that have been identified as cell-binding regions.


A
H2N
30k
40k
20k
75k
35k
30k
COOH
S S
S S
H2N
30k
40k
20k
75k
33k
30k
COOH
Heparin I
Fibrin I
Collagen
Fibrin II
Cell
Heparin II
Fibrin III

The best characterized site on the fibronectin molecule, is the cell-binding domain. By successive shortening of the 75 kDa fragment that conserved the cell-binding ability, it was found that the tetrapeptide, Arg-Gly-Asp-Ser (RGDS) is the minimal sequence required to permit cell attachment to fibronectin-coated substrata (Pierschbacher & Ruoslahti, 1984a). This sequence is found in a segment of fibronectin that would be expected to form a hydrophilic loop at the surface of the molecule, and would, therefore, be available to interact with cells (Pierschbacher & Ruoslahti, 1984a). Peptides containing this sequence promote cell-attachment when insolubilized on a surface, but competitively inhibit the attachment of cells to fibronectin, when they are in a soluble form.

Two factors to take note of at this point, are, firstly, results indicate that conservative substitutions in the first three amino acids, abolish activity, whereas the serine residue appears not to be essential to cell-attachment, although only conservative substitutions, such as threonine, alanine, valine, and cysteine, are compatible with activity (Pierschbacher & Ruoslahti, 1984b). Interestingly, RGDX sequences, where X can be one of the aforementioned amino acids, have been found in other proteins with cell-binding activities, such as collagen (RGDT, RGDA), fibrinogen (RGDS) (Pierschbacher & Ruoslahti, 1984b), thrombospondin (RGDA), and vitronectin (RGDV), also known as serum-spreading factor (Yamada & Kennedy, 1987). In addition, laminin also contains the RGD sequence (see section on laminin). The second point to note, is that sequences flanking the RGDS sequence can also affect its binding activity (Pierschbacher & Ruoslahti, 1984a). The most effective small peptide is the pentapeptide GRGDS (Yamada, 1989).

The affinity of synthetic peptides, or even the 11.5 kDa cell-binding fragment for fibroblasts, is substantially lower than larger fragments, or the intact fibronectin molecule (Akiyama & Yamada, 1985; Akiyama *et al.*, 1985). This would suggest that, although the RGDS sequence is the minimum cell-recognition sequence on fibronectin, sequences outside this site must play an important role in the efficient binding of fibronectin to cells (Akiyama *et al.*, 1985). This led to the discovery of a number of other potential cell-binding sites. Humphries *et al.*, (1986), characterized a site which possessed cell-type specificity. The sequence, Arg-Glu-Asp-Val (REDV) was found to be inhibitory for melanoma cell-adhesion, but inactive for fibroblast cell-adhesion. This particular sequence, termed CS5, however, is found in human, but not chicken fibronectin. A second site, termed CS1, was also located by Humphries *et al.*, (1987), and it is thought to function in an additive manner with the REDV site. Recently, a third cell-binding site has been identified, which functions synergistically with the RGDS site (Obara, 1987; Obara, 1988). Any mutational inactivation of either of these sites leads to, at least, a 95% loss of cell adhesive activity (Obara, 1988).

Dufour *et al.*, (1988) demonstrated with avian neural crest cells, that the RGDS site (including the synergistic site) and the CS1 site were able to promote attachment of the cells, but only the RGDS site could support their spreading. Without the synergistic site, the RGDS domain was unable to promote attachment, or spreading. Finally, all three domains *ie.* RGDS and the synergistic site, plus CS1, were required, in association, for cell motility. These results indicate that the RGDS sequence alone, is not sufficient to promote attachment, spreading and motility of neural crest cells, but instead, requires the presence of the synergistic adhesion site. Although not directly related, but in keeping with this thought, it has recently been established that cell surface glycolipids are associated with the fibronectin receptor (Cheresh, 1987a; Cheresh *et al.*, 1987). It is possible that the role of the glycolipid may be to orient the divalent cation that is required for the receptor to bind to its ligand, thereby increasing the avidity of the cell-fibronectin interaction. Another possibility may be that the glycolipid provides the initial non-specific electrostatic attraction between the cell and the substratum, until the interaction is complete. In this way, depending on the particular oligosaccharide moiety, a glycolipid may provide a preferred orientation for optimal receptor-ligand interaction. This would suggest that glycolipids with different compositions could provide cells, with identical RGD-dependent receptors, with varying affinities for a given substrate.

From the information gathered to date, it appears that the RGDS site alone, is not sufficient, but requires the interaction of a number of other factors, including additional binding sites, divalent cations, and other cell-surface interactions, to achieve a full range of cellular activities.

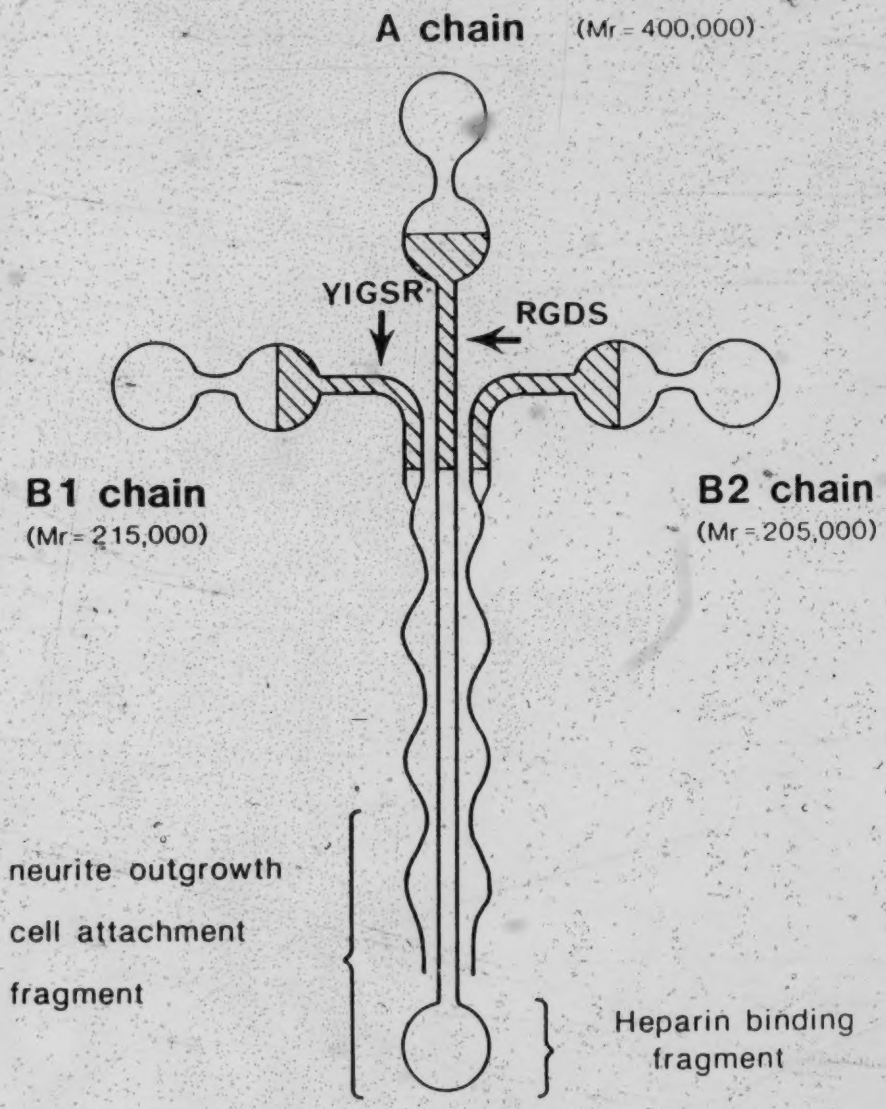
LAMININ

Laminin was first isolated from the mouse Engelbreth-Holm-Swarm (EHS) tumor (Timpl *et al.*, 1979). Its effects, primarily, are targeted at cells of epithelial origin. Laminin has been implicated in many important cellular functions, including cell adhesion (Terranova *et al.*, 1980; Couchman *et al.*, 1983), neurite outgrowth (Manthorpe *et al.*, 1983), morphogenesis (Palm & Furcht, 1983), and differentiation (Grover *et al.*, 1983), in addition to providing an important structural component for basement membranes.

Rotary-shadowing electron microscopy reveals a structure, cruciform in shape, with one long arm of 77nm in length, and three short arms of 36nm in length (Engel *et al.*, 1981). Each molecule consists of three polypeptide chains, designated A, B_1, and B_2, with a total molecular weight of 900kDa. Two small globular domains are observed at each end of the two short chains, and one larger globule at the end of the long arm. The three chains intersect at the centre of the cross, are linked by disulphide bonds, and then run together, parallel, to form the rod-like segment of the long arm (see Figure 2 for primary structure).

The general description of laminin is based on the structure of the glycoprotein isolated from the EHS tumour. Variations in the structure have been found in laminin purified from invertebrate tissues, such as *Drosophila* (Fessler *et al.*,

Figure 2 - primary structure of the laminin molecule, showing the YIGSR, and RGDS cell-binding regions, in addition to regions that have been shown to be involved in heparin-binding and neurite outgrowth.


A chain
(Mr = 400,000)
YIGSR
RGDS
B1 chain
(Mr = 215,000)
B2 chain
(Mr = 205,000)
neurite outgrowth
cell attachment
fragment
Heparin binding
fragment

1987), sea urchin embryos (McCarthy *et al.*, 1987), and leech (Chiquet *et al.*, 1988), and quite recently, S-laminin, isolated from the synaptic cleft of the neuromuscular junction (Hunter *et al.*, 1989), mouse heart laminin (Paulsson & Saladin, 1989), and merosin (Ehrig *et al.*, 1990). Embryonic forms of laminin appear to consist of only the B chains, whilst the A chain is not detectable until later on in development (Cooper & MacQueen, 1983). The A chain also appears to play a role in the development of epithelial cell polarity (Klein *et al.*, 1988). In the embryonic kidney, non-polarized mesenchymal stem cells convert into epithelial cells, as a response to an inductive cell-cell interaction. It was suggested that the appearance of the A chain (occurring due to this induction) leads to a deposition of laminin into the basal extracellular matrix, which, in turn, triggers epithelial cell polarization. All the variations detected to date, show preservation of the length of the two short arms of the molecule, but the length of the long arm appears to vary considerably with the species. Variations such as this, may be required to span basement membranes of different thicknesses (Beck *et al.*, 1990)

Like fibronectin, laminin is able to bind with a number of other proteins, including collagen type IV, nidogen, and heparin. It forms a particularly stable complex with nidogen, and can be found in many tissue extracts to exist in equimolar proportions with the nidogen molecule (Dziadek & Timpl, 1985). Nidogen has been demonstrated to bind to type IV collagen, and thus, may provide a means of attachment between laminin and type IV collagen (Aumailley *et al.*, 1989). Laminin, however, also has the ability to bind to type IV collagen directly, via the globular regions of either of its four arms (Charonis *et al.*, 1985; Laurie *et al.*, 1986). A major binding site for heparin was localised to the terminal globule of the long arm (Ott *et al.*, 1982), and may also be involved in the binding of basement membrane heparan-sulphate proteoglycans.

Of great importance, at least with regard to this study, is the ability of laminin to bind to cells. The pentapeptide, Tyr-Ile-Gly-Ser-Arg (YIGSR), which is present in the B_1 chain, was the first cell-binding region to be detected, and was found to be active in both cell adhesion and migration (Graf *et al.*, 1987a; Graf *et al.*, 1987b). In addition to this site, two more sites have been found to be involved in cell attachment and migration (Charonis *et al.*, 1988; Tashiro *et al.*, 1989). Until recently, it was thought that the sequence YIGSR was only present in laminin, and the RGDS sequence only present in fibronectin. In 1989, however, Grant *et al.*, showed that laminin did indeed possess an RGD sequence, in the short arm of the A chain, and that this sequence was also active, as in fibronectin, in cell attachment. (see Figure 2). In experiments with cultured endothelial cells, Grant *et al.*, (1989) were able to demonstrate that each peptide had a separate activity. The primary role of the RGD-containing peptide was in the initial attachment of the cells to the substratum, whereas the YIGSR site was involved in cell differentiation.

Although there has been less investigation into the function of laminin, in comparison to fibronectin, its appearance early on in development, suggests that it may play an important role in the migration of the mesoderm cells during gastrulation, although its exact function at this time, has not yet been determined.

INTEGRINS

The attachment of cells to their environment is important in determining cell shape, and in the maintenance of proper cell function and tissue integrity. Most cells possess a variety of mechanisms for attaching to the surrounding extracellular matrix and neighbouring cells, including peripheral membrane glycoproteins, glycosyltransferases, and proteoglycans, in addition to cell junctions. A large family of transmembrane glycoprotein receptors have recently been discovered to be involved in cell adhesion, and these have been termed integrins.

Each integrin, with the exception of the complex dissociated from chicken cells, is a heterodimer composed of a more conserved ß chain (95 - 130kDa), noncovalently associated with one of several distinct α chains (130 - 210kDa; Hynes, 1987). When visualised by a variety of electron microscope methods, purified, detergent-solubilized receptors appear to be composed of a globular head with two relatively long tails (Carrell, 1985; Kelly *et al.*, 1987). To date, six ß chains have been identified (Sonnenberg *et al.*, 1990). The first three subunits possess 45% homology in their primary amino acid sequence, and define one of the three integrin subfamilies. The first group ($ß_1$) includes the VLA antigens (very late activation antigen of T-cells; Hemler *et al.*, 1987), consisting of five members, including the human fibronectin receptor (Pytela *et al.*, 1985), and the CSAT (chicken fibronectin-laminin receptor - Horwitz *et al.*, 1985). The second, or $ß_2$ group, includes the leukocyte adhesion molecules (Springer, 1985), and the third group ($ß_3$) are the cytoadhesins, which include the vitronectin receptor, and the platelet glycoprotein IIa-IIIb. The vitronectin receptor binds to vitronectin, von Willebrand factor, fibrinogen (Cheresh, 1987b), and thrombospondin (Lawler *et al.*, 1988), whilst the platelet glycoprotein is a receptor for fibronectin (Plow *et al.*, 1985), vitronectin (Pytela *et al.*, 1986; Thiagarajan & Kelly, 1988), von Willebrand factor (Ruggeri *et al.*, 1983), and fibrinogen (Bennett *et al.*, 1982). Quite recently, a fourth class of ß subunit was identified, in the *Drosophila* position specific antigens (Leptin *et al.*, 1987), mouse mammary tumor cells (Sonnenberg *et al.*, 1988), colon carcinoma cells (Hemler *et al.*, 1989), and both normal and malignant epithelial cells (Kajiji *et al.*, 1989).

The α subunits, which appear to confer binding specificity, are homologous with one another, although apparently not with the ß subunits. Current evidence indicates that there are at least eleven different α chains: six associated with $ß_1$, three with $ß_2$, and two with $ß_3$. This does not rule out the possibility, however, that there are other α chains, or that a particular α chain can associate with more than one ß chain (Hynes, 1987). Both the α and ß subunits are, as previously mentioned, transmembrane glycoproteins, with a large amino-terminal extracelluar domain, a single hydrophobic transmembrane sequence, and a short carboxy-terminal cytoplasmic domain (Buck & Horwitz, 1987). The α subunit differs slightly, in that its long 'arm' consists of two disulphide linked polypeptides, whereas the smaller chain contains the transmembrane and intracellular domains. Although integrins from different cell types may consist of the same α and ß chains, and appear to be immunologically indistinguishable, it has been shown that they can differ significantly in their binding to various ligands (Kirchhofer, 1990). These results could be interpreted to mean that different cell types receive different signals from the same type of extracellular matrix *ie.* regulation is by cell-type specific factors. Most integrins require divalent cations, such as calcium for the interaction with their ligand. It has not yet been determined, however, whether the cations are required for the ligand-receptor interaction, or stabilization of the α-ß interaction (Cassiman, 1989).

The general function of integrins, is in cell adhesion. Many were identified by their ability to bind extracellular matrix glycoproteins. The RGD sequence has been shown to be present in a number of these glycoproteins (see Ruoslahti & Pierschbacher, 1987, for review), and it is now known that many of the integrins bind proteins containing this sequence (see Hynes, 1987, for review). In fact, they are also able to recognise differences in the conformation of the RGD peptide, including substitutions of L-arginine with D-arginine (but not L-aspartic acid with D-aspartic acid) and the inverted fibronectin peptide, SDGR, although to a lesser extent (Pierschbacher & Ruoslahti, 1987; Yamada & Kennedy, 1987).

One of the first integrins to be identified and characterized as a possible receptor for extracelluar molecules, was the 140kDa, oligomeric complex from avian cells, or CSAT (cell substratum attachment antigen; Neff *et al.*, 1982; Horwitz *et al.*, 1985). Unlike other integrins, CSAT consists of three, not two, glycoproteins, with a molecular weight of 120 - 160 kDa. Using both monoclonal and polyclonal antibodies, it was possible to show the distribution of this integrin in fibroblastic and other cell types (Damsky *et al.*, 1985; Chen *et al.*, 1985). On well-spread, stationary fibroblasts, integrin is found along portions of stress fibres, and at their termini, in focal contacts. The latter are regions of close apposition between the cell membrane and the substratum, and are characterized on the cytoplasmic side, by the termination at this point, by bundles of microfilaments. This localization of integrin corresponds with the distribution of areas enriched with cytoskeleton-associated molecules, such as vinculin, talin, and α-actinin, which are thought to serve as links between the cell surface and the actin stress fibres, and may indicate a possible function for integrin in mediating cell-matrix adhesion. Integrin also localises at the surface of cells that are in contact with the substratum, which is also where extracellular fibronectin fibrils can be seen. Experiments similar to those just described, have shown a co-localization of integrin and talin at cell membranes adjacent to fibronectin-coated beads (Mueller *et al.*, 1989). Integrins on migrating cells, however, are diffusely distributed over large regions of their surfaces (Damsky *et al.*, 1985; Duband *et al.*, 1986). Such experiments demonstrate that integrin is an important link between the ECM, and the cytoskeleton of the cell, and that the integrin-talin coaggregation can initiate cytoskeletal events necessary for cell adhesion and spreading. Integrins may well be involved in other processes, for example, phagocytosis (Gresham, 1989), but further study in these areas is still required.

USE OF SYNTHETIC PEPTIDES IN DEVELOPMENTAL STUDIES

Morphogenetic events in embryonic development appear to require the precise sequential functioning of specific macromolecules, and in many instances, these molecules may be developmentally regulated. To demonstrate directly the specific roles for particular molecules, however, has been arduous, since ablation of the particular molecule is required, to show that it is causal in the process being studied.

One approach to this problem, is the use of antibodies to inhibit the function of the molecule of interest. This method has been used in numerous experiments, but suffers from certain limitations. Firstly, it is not always possible to prove directly that the antibody is indeed inhibiting or ablating the function of the required molecule. Secondly, the effects seen, could be due, indirectly to a cross-reaction of the antibody with another molecule, or even caused by the antigen-antibody complex itself.

An alternative to the use of antibodies, in some instances, would be the use of synthetic peptides, which competitively inhibit the function of the observed molecule (Akiyama & Yamada, 1985). This would provide a direct means of inhibition, in addition to the fact that peptides would be expected to diffuse more readily than antibodies, through tissues, since they are somewhat smaller (Boucaut *et al*, 1984b).

Boucaut *et al.*, (1984a), injected antibodies against fibronectin into the amphibian embryo, *Pleurodeles waltlii.* They were able to demonstrate that gastrulation was inhibited by the antibodies, but not neurulation. This would indicate that fibronectin was important for the migration of the mesoderm during gastrulation, but not as important for the movement of the cells during neuralation. In the same year, Boucaut *et al.*,(1984b) injected a decapeptide containing the cell-adhesion site from fibronectin, into both amphibian and avian systems. Amphibian embryos injected at the blastula stage of development with this peptide, displayed a strikingly abnormal morphology, and no migrating cells were found along the basal surface of the ectoderm, of the blastocoel roof. Neural crest cultures from avian embryos, when treated with the same peptide, became detached from their substratum and rounded up. Neural crest cells *in vivo* were prevented from migrating forwards, along their usual pathway, when the peptide was added. A valid point to note here, is that although part of the sequence of the peptide that was injected can be found in a few other proteins (Pierschbacher & Ruoslahti, 1984a), the full decapeptide sequence appears to be unique, thereby ensuring that only the function of the required molecule is inhibited.

Another study using synthetic peptides was conducted by Lash *et al.*, (1987). The purpose of the study was to investigate the effect of fibronectin-derived synthetic peptides upon cell-cell and cell-substratum adhesion in distinctive embryonic cell types, including isolated cells from somites and segmental plates, and upon tissue migration, using precardiac mesoderm. They found that dissociated segmental plate cells, which normally exhibit little tendency to adhere to each other, or to the substratum, on the addition of the peptide GRGDS (more active that RGDS in fibroblast adhesion assays - Yamada & Kennedy, 1985) cell-cell adhesion was dramatically stimulated, whilst the already minimal cell-substratum adhesion was completely inhibited. The somitic cells, which normally exhibit a strong tendency to adhere to one another and to the substratum, were inhibited from adhering to the substratum, when GRGDS was added, although cell-cell adhesion was slightly enhanced. This slight enhancement of adhesion was hypothesized to be the result of the peptide acting as a signal or trigger on the cells within the somite, to initiate the process of somitogenesis. Neither the segmental plate cells, nor the somitic cells showed a response to peptides that did not contain the RGD sequence. Under the experimental conditions used in these particular experiments, the precardiac mesoderm showed a tendency to spread on tissue culture substratum, particularly fibronectin-coated substratum. In the presence of the pentapeptide GRGDS, tissue spreading was greatly inhibited, and both cell-cell contact, and cell-substratum interactions were affected.

The role of fibronectin and laminin in the migration of the Wolffian duct of avian embryos has also been examined using the synthetic peptides GRGDS and YIGSR. After microinjection of the former peptide, there was a compaction of the mesenchymal cells at the tip of the Wolffian duct, and an inhibition of their normal migration (Jacob *et al.*, 1989). Injection of RGD-containing peptides into *Drosophila* embryos have been shown to prevent gastrulation (Naidet *et al.*, 1987). More recently, the same pentapeptide has been employed in the chick embryo, to investigate the possible role of fibronectin in the migration of the mesoderm cells at the edge of the blastoderm, on the vitelline membrane (Lash *et al.*, 1990). Using the technique invented by New (1955), Lash *et al.*, treated the embryos with a number of different peptide sequences. The peptide GRGDS was found to cause the edge cells of the blastoderm to detach from the vitelline membrane, and the expansion of the blastoderm was inhibited. Using scanning electron microscopy, it was observed that the cells at the margin of the blastoderm had lost all cell processes, and, as a direct consequence, the cells became detached from the vitelline membrane, and the blastoderm ceased to spread. Immunocytochemical staining with anti-fibronectin demonstrated that fibronectin was present at the interface of the edge cells and the vitelline membrane, and also between the epiblast and hypoblast. These results indicate, not only that the tissue movement during blastoderm spreading is dependent on fibronectin, but in addition, that the specific RGD sequence and its associated integrin receptors, are important factors contributing to this embryonic morphogenetic movement.

It appears, therefore, that synthetic peptides may provide an valuable tool for the manipulation of both *in vivo*, and *in vitro* environments, especially during embryogenesis, when the interactions between cells and substrates are important for the synchronized development of the embryo. In addition, they may eventually useful in therapeutic roles, for example, in counter-acting the invasion of tumor cells through tissues (Gehlsen *et al.*, 1988).

MATERIALS AND METHODS

IN VIVO EXPERIMENTS

PREPARATION OF EMBRYOS

Fertilized eggs of White Leghorn chickens were purchased from the Poultry Farm, University of Alberta. The eggs were incubated for 17 hours at 40°C, after which the yolk was removed, and placed in Pannett and Compton's saline (PCS; (Pannett & Compton, 1924).

Pannett and Compton's saline, a buffered isotonic saline, was prepared by mixing 60.55g NaCl, 7.75g KCl, 3.85g $CaCl_2$, and 6.35g $MgCl_2.6H_2O$ in 500mls of dH_2O (solution A), and 0.945g Na_2HPO_4, and 0.095g $NaH_2PO_4.2H_2O$ also with 500mls of dH_2O (solution B). These two stock solutions were stored in the refrigerator at 4°C until required. PCS was prepared just prior to use, by combining 40mls of stock solution A and 60 mls of stock solution B, with 900mls of sterilized ddH_2O.

WHOLE EMBRYO CULTURES

The yolk, still in PCS, was cut in half, at its equator, and the piece of vitelline membrane with the attached embryo was removed. It was then placed in a watch glass, with the embryo surface-side uppermost. A glass ring, with an internal diameter of 25mm, was placed on top of the vitelline membrane, so that it encircled the embryo. The edges of the membrane were then pulled taut, over the ring, and the excess trimmed away (New, 1955). Successive washes with PCS were made until all the remaining yolk droplets had been removed from the culture. Albumin, previously collected when the egg was opened, was pipetted under the vitelline membrane, displacing any PCS. This acted as a natural bactericide.

MICROINJECTION OF EMBRYOS

The embryos were left in the incubator, at 40°C for 1 - 1 $^1/_2$ hours, to ensure that the blastoderm adhered securely to the vitelline membrane, after which, they were removed, and injected with either a peptide, or antibody. The injection set-up consisted of a dissecting microscope, and a three-way movable micromanipulator, to which the pipette was attached. The pipette was pulled using a Narishige pp-83, two-stage electrode puller, and broken to the required 20 - 30μm (determined by measurement with a slide micrometer) using forceps. The pipette was then inserted into the end of a length of plastic tubing, via a specially designed end-piece containing a ring of rubber tubing. This provided a seal between the pipette, and the tubing, and maintained the internal pressure. At the end of the tubing, a 1ml syringe was attached. Using the micromanipulator, the pipette was manouvered until it pierced the endoderm to the right of the primitive streak at approximately the mid-way point of the embryo (Figure 3), and approximately 200nl of the experimental solution was injected into the mesodermal space, using pressure generated by the syringe. This volume was estimated by injecting equivalent amounts of methylene blue stain into paraffin oil, and measuring the diameter of each bubble that was formed. Using the formula, Volume $= 4/3\pi r^3$, an estimate was made of the volume of the solution injected. Preliminary experiments involved the injection of blue-dyed polystyrene beads, 5.53 μm in diameter (Polysciences Inc.) Further experiments injected medium containing the peptides, RGDS (n=19; Peninsula Laboratories Inc., or Sigma Chemical Co.), GRGESP (n=21; Peninsula Laboratories Inc., or Bachem Inc.), and YIGSR (n=9; Peninisula Laboratories Inc.) at a concentration of 10^{-2}M. Rabbit α-human fibronectin (n=18; Collaborative Research Inc.), and rabbit α-mouse laminin antiserum (n=17; Collaborative Research Inc.) were injected at a dilution of 1:40 in normal medium. PCS (n=22) was also injected, as a control for the YIGSR peptide. The cultures were then returned to the incubator at 40°C for a further 6 hours.

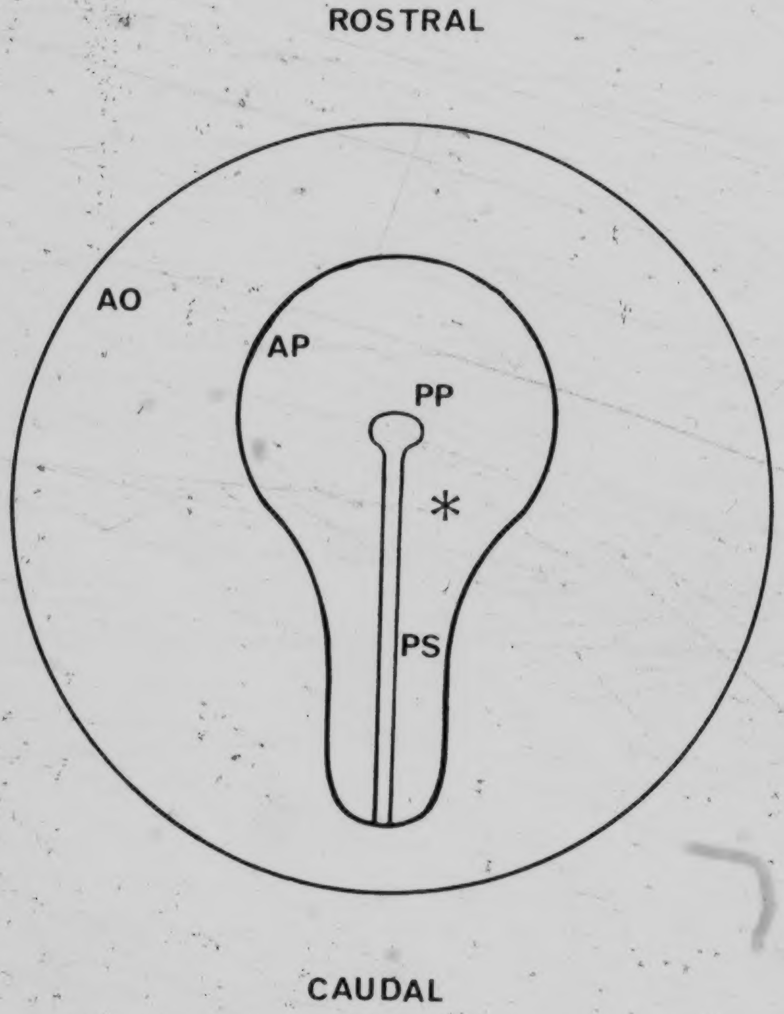
Figure 3 - illustration of a stage 4 chick embryo indicating the approximate region for microinjection of experimental solutions (*).

AO - Area opaca

AP - Area pellucida

PP - Primitive pit

PS - Primitive streak


ROSTRAL
AO
AP
PP
*
PS
CAUDAL

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Following exposure to the experimental solution, the embryos were fixed overnight, in 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4 (Sorensen, 1909), and kept in the refrigerator, overnight at 4°C. They were then washed in 0.1M phosphate buffer pH 7.4 (3 x 10 minutes), and post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer pH 7.4 for one hour. This was followed by another wash in 0.1M phosphate buffer (3 x 10 minutes), and then dehydration in a graded series of ethanol (10 minutes in each of 50%, 70%, 80%, 95%, and twice in absolute ethanol). The specimens were then immersed in propylene oxide for 30 minutes, before being placed in a 1:1 mixture of propylene oxide and Araldite embedding resin (Luft, 1961). The specimens remained in this mixture overnight at room temperature. The embryos were then removed, and placed in embedding molds, already half full of polymerised Araldite. The molds were then completely filled with liquid Araldite, and placed in an oven at 60°C for 48 hours. The blocks were removed from the molds, and trimmed with a razor blade.

Thin sections showing a gold interference pattern (approximately 90nm) were cut, using glass knives on either a Reichert-Jung Ultracut or a Reichert Om U2 microtome. Sections were picked up on formvar-coated 200 mesh copper grids. Sections were stained with 5% uranyl acetate in absolute methanol for 10 minutes, washed in 4 changes of absolute methanol, and counter-stained in Reynold's lead citrate (Reynolds, 1963) for 60 seconds. Grids were examined with a Phillips EM 300 transmission electron microscope, at 80 KV.

LIGHT MICROSCOPY

The same procedure was followed for light microscopy, up to, and including block trimming. Thick sections were cut, using glass knives, of approximately 1 μm on either a Reichert-Jung Ultracut or a Reichert Om U2 microtome. The sections were then placed on glass microscope slides, dried, and stained with Richardson's stain on a hotplate preheated to 200°C. Richardson's stain was made by mixing 1 part Azure B to 1 part 1% Methylene blue in 1% borax, and then filtered. Stained sections were viewed and photographed using a Leitz microscope.

SCANNING ELECTRON MICROSCOPY (SEM)

Embryos were prepared in the same way as TEM, up to the point of ethanol dehydration. Then instead, embryos were passed through a graded series of acetone (10 minutes in each of 50%, 70%, 80%, 95%, and absolute acetone), and stored in absolute acetone until they were critical-point dried using CO_2. Specimens were then split across the primitive streak, using tungsten needles, and mounted on stubs using conductive paint, so that the broken edge, displaying the primitive streak, was visible. Specimens were then sputter-coated with gold, and examined on a Phillips 505 scanning electron-microscope.

IN VITRO EXPERIMENTS

TISSUE CULTURE

Eggs were incubated for 24 hours at 40°C to obtain Stage 5 (Hamburger and Hamilton, 1951) embryos. The embryos were removed from the overlying vitelline membrane, and placed in Tyrode's saline pH 7.4, an isotonic saline solution.

~~Tyrode's~~ saline was prepared, by mixing 0.6g $CaCl_2$, 0.3g $MgCl_2.6H_2O$, 0.6g KCl, 3.0g $NaHCO_3$, 24.0g NaCl, 0.15g $NaH_2PO_4.H_2O$, and 3.0g glucose with 3 litres of ddH_2O. The pH was adjusted to 7.4, using 10% NaOH, or 1N HCl, and then passed through a millipore filter (pore size, 0.22μm).

Using sterile technique and tungsten needles the endoderm layer was removed, and mesoderm cells were scraped away, and collected in a separate Falcon dish. The cells were then transferred in a few drops of Tyrode's saline and placed on glass coverslips via silicon-coated narrow-mouth Pasteur pipettes. In some instances, the glass coverslips were coated with a layer of fibronectin, or laminin, at a concentration of 1mg/ml.

To coat a batch of coverslips, 25μl of fibronectin or laminin was mixed with 0.5mls of normal medium with gentamycin. A few drops of this solution was then placed on each coverslip and left for approximately 45 minutes. The coverslips were then washed, with 199 medium, to remove any excess fibronectin or laminin.

Once the mesoderm cells had been transferred to the coverslips, the excess Tyrode's saline was removed with a narrow-mouth pipette, and replaced with 199 medium containing 5% Fetal Bovine Serum (Gibco Laboratories), and gentamycin sulphate solution, at a concentration of 10μg/ml. The fibronectin had been removed from the serum, by passing it through a gelatin-sepharose column. In addition to this, in some experiments, various peptides and antibodies were also added. RGDS (Peninsula Laboratories Inc., or Sigma Chemical Co.), GRGESP (Peninsula Laboratories Inc.), and YIGSR (Peninsula Laboratories Inc.) were used at a final concentration of 5 x 10^{-3}M, and polyclonal anti-fibronectin, (Collaborative Research Inc.) anti-laminin antiserum (Collaborative Research Inc.), Rabbit IgG (Jackson Immunoresearch Laboratories Inc.), and α-fibronectin receptor antibodies (Calbiochem Corporation) were added at a dilution of 1:40 with normal medium. The particular concentrations used, were determined after a range of initial dilution experiments were carried out. The cultures were then placed in a 5% CO_2

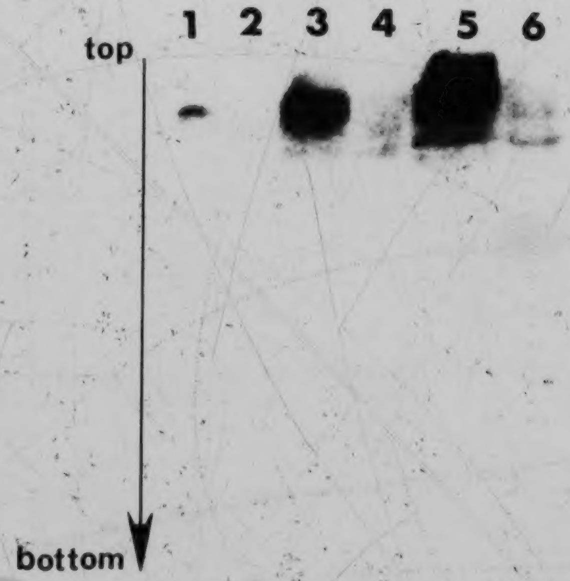
environment, and incubated at 40°C for 24 hours. They were then viewed and photographed, using a Nikon inverted phase-contrast microscope.

Removal of fibronectin from fetal bovine serum

Regular fetal bovine serum was run through a gelatin-sepharose column, and the eluent was collected. To ensure that the serum was indeed, free of fibronectin, samples of both the regular serum, and the fibronectin-free serum were run at 1μl, 8μl, and 25μl amounts on a Western Blot (courtesy of Dr. Ernst Streyer and Dr. Wolfgang Schneider). Anti-fibronectin antibodies (20μl in 7μl of 5% skimmed milk) were used to label any fibronectin that was present, and the gel was visualised using ^{125}I-Protein A in 5% skimmed milk, for 18 hours (Figure 4).

Results indicated, at each amount used, that there was no fibronectin present in the serum that had been run through the gelatin sepharose column.

Figure 4 - Western blot comparing the fibronectin content of normal serum, (lanes 1, 3, and 5), with serum that was run through a gelatin-sepharose column (lanes 2, 4, and 6). Lanes 1 & 2 show 1μl of serum, lanes 3 & 4 show 8μl of serum, and lanes 5 & 6 show 25μl of serum. Note the absence of fibronectin in the serum that was run through the column.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MEASUREMENTS AND QUANTITATION

LIGHT MICROSCOPY

Stained slides were viewed using a Leitz microscope, at x10 and x25 magnification. A micrometer was used to measure the width of the embryo, the distance between the mesoderm and ectoderm, and the distance between the mesoderm and endoderm, at predetermined points, and the number of cells attached to the basement membrane of the ectoderm. The points used for obtaining measurements, were determined at x25 magnification by orientating the transverse section longitudinally, and aligning the primitive streak centrally within the field of view. Distances between the various tissue layers were measured at the extreme top and bottom of the field of view, and the number of mesoderm cells attached to the basement membrane within the same area, were counted. These values were then used in statistical comparisons.

SCANNING ELECTRON MICROSCOPY

The exposed region of the primitive streak was examined, and electron micrographs of cells subjected to the various treatments, were taken. Those cells whose outlines were visibly uninterrupted, were put to one side, and subsequently used for the 'shape' analysis. Magnifications on the negatives ranged from x274 to x2020, and prints used for the analysis, were made at either x3 or x4 the size of the negative. A Summagraphic digitizing pad (Summagraphic Corporation, Seymour, Connecticut) was interfaced to an IBM personal computer, and the 'Bioquant 3D' analysis program was used (R & M Biometrics Inc., Nashville, TN), to trace the outlines of the chosen cells (courtesy of Dr. Rex Holland). This allowed computation of a shape factor for each tracing, a value between 0 and 1, where 1 is equal to a perfect circle. Although the magnifications were not consistent, and the cells, therefore, appeared to vary in size, this did not affect the values obtained from the cell tracings. The same cell, traced at two different magnifications, showed approximately equal values for each analysis. The same procedure was followed for all the *in vivo* microinjection experiments, and the values obtained, were used for statistical comparisons between the various treatments.

TISSUE CULTURE

Cell cultures were viewed using a Nikon inverted phase-contrast microscope. Experiments in which cells were grown on glass coverslips, or glass coverslips coated with fibronectin, and grown in either normal medium, or medium containing RGDS, were subject to quantitation. All cell types falling within the field of view, were counted. This procedure was followed for 1) fibroblastic cells on glass coverslips with normal medium, 2) epithelial cells seen on fibronectin-coated coverslips with normal medium, 3) rounded cells seen on glass coverslips with RGDS-containing medium, and the assorted morphology of cells observed on fibronectin-coated coverslips with RGDS-containing medium. The data obtained was used in a graphical illustration, depicting the range of cell shapes observed under various experimental conditions (Figure 28 - see results for explanation).

RESULTS

GENERAL MORPHOLOGY OF THE STAGE 5 CHICK EMBRYO

By stage 5 (Hamburger & Hamilton, 1951), the primitive streak has transversed two-thirds of the way across the area pellucida, and has reached its maximum length, before it begins to regress (Figure 5). The ectoderm is moving towards the midline, where it invaginates at the point of the primitive streak, displaying the characteristic "flask-shaped" cells (Figure 6a, 6b), and, ultimately differentiates into endoderm, or mesoderm, the latter migrating laterally between the two developing layers. Figure 7 shows the mesoderm migrating between these two layers, and clearly illustrates the elongated cells of the upper ectoderm, and the individual cells of the lower, single layer endoderm. At this stage, the mesoderm cells possess many filopodia and lamellipodia which they use to attach to other cells, and the basement membrane. The filopodia can be seen as long cellular processes that extend outwards from the cell, where they make contact with another cell, or the ectoderm or endoderm (Figure 16a). The lamellipodia, however, are more flattened cellular processes, that appear to be involved in the actual forward migration of the cell along the basement membrane (Figure 16b). High power transmission electron micrographs reveal that the mesoderm cells actually make contact with the basement membranes of the ectoderm during their migration. Figure 8a shows a single

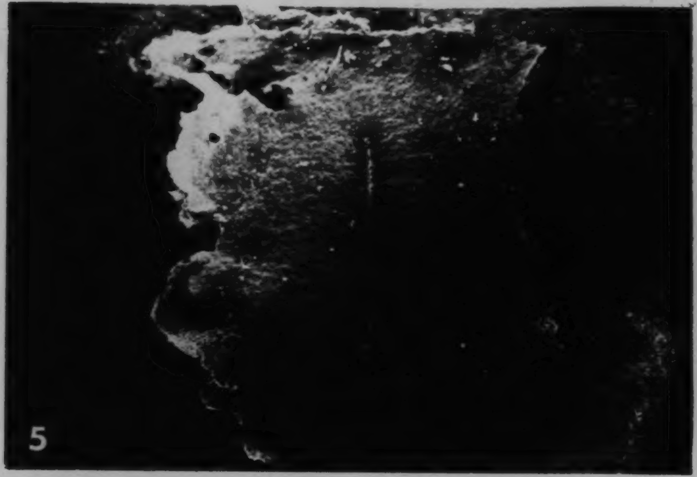
Figure 5 - SEM of a stage 5 chick embryo, showing area pellucida (area opaca has been removed) with the primitive streak extending rostrally (towards the top of the photograph). (x984).

Figure 6a - SEM of the primitive streak region of a stage 5 chick embryo, showing the ectoderm (ec), and endoderm (en), with the mesoderm (m) between them. (x2775).

Figure 6b - higher power magnification (SEM) of the primitive streak region showing the invagination of the ectoderm. Note the contact between the mesoderm and ectoderm. (x3450)

ec - ectoderm; m - mesoderm; en - endoderm; ps - primitive streak

Figure 7 - SEM of a stage 5 chick embryo showing the mesoderm cells (m) between the ectoderm (ec), and the endoderm (en). Note the elongated cells of the ectoderm, the fibroblastic morphology of the mesoderm, and the single cell, sheet-like layer of the endoderm. (x936).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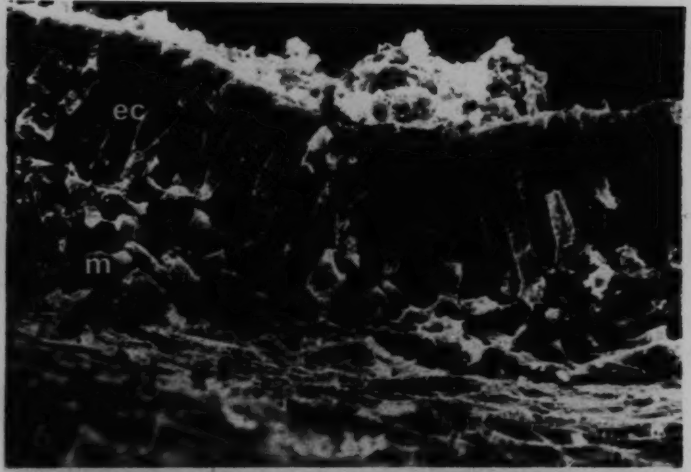
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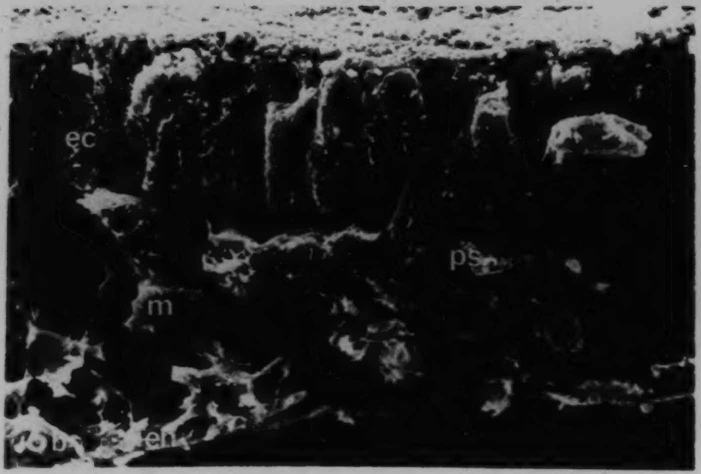
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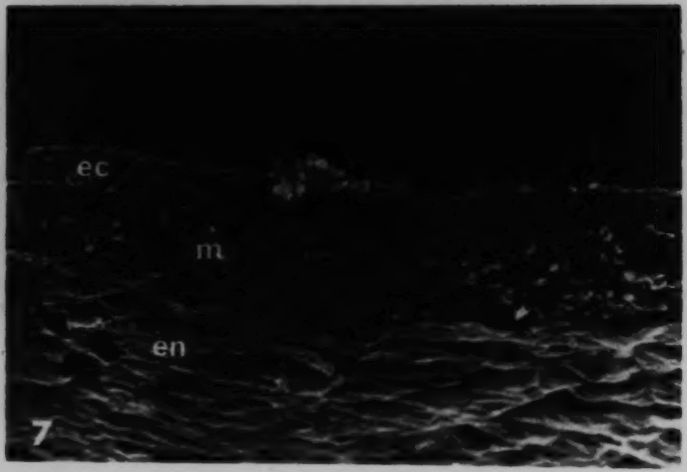

ec
m
en
7

mesoderm cell in contact with the basement membrane of the ectoderm. Also visible in this micrograph, is an interstitial body. These are dense, granular structures (Bellairs, 1963; Low, 1970), which have been shown to be rich in fibronectin (Sanders, 1982). Although they are often associated with basement membranes, the origin and function of the interstitial bodies is, as yet, unknown. Figure 8b shows a higher power magnification of the interaction between a mesoderm cell, and the ectoderm, and clearly shows both the lamina lucida and the lamina densa of the basement membrane.

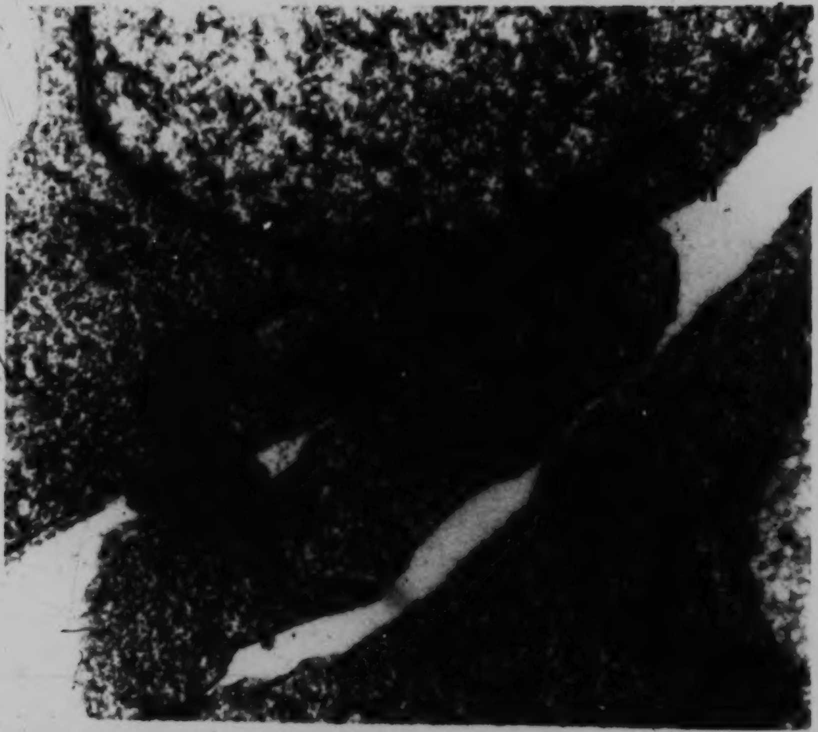
IN VIVO EXPERIMENTS

Embryos injected with either a peptide or antibody, were treated in a number of ways. Some were embedded in araldite, and sectioned for light microscope analysis, whilst others were prepared for either transmission electron microscopy or scanning electron microscopy. Observations of the embryos using TEM, however, proved to be too high power to enable the visualization of any gross morphological changes. Initial experiments involved the injection of polystyrene beads into the space between the ectoderm and endoderm, to ensure that it was indeed possible to successfully inject into this area (Figure 9). Transverse sections of the embryo showed a localization of beads within the space between the ectoderm and endoderm, predominantly on the same side of the primitive streak as the injection site.

Figure 8a - TEM of a mesoderm cell migrating on the basement membrane of an ectoderm cell. (x38,160)

bm - Basement membrane; m - Mesoderm cell; ib - Interstitial body

Figure 8b - Higher power magnification (TEM) of a mesoderm cell (m) migrating on the basement membrane of an ectoderm cell (ec). Note the two visible layers (lamina lucida, and lamina densa) of the basement membrane (bm). (x31,601).



8b

Occasionally, a few beads were found on the outside of the embryo, but this was only due to a slight leakage of the beads immediately after the injection.

Once an araldite block had been sectioned and stained, one slide per embryo was used for measurements. Embryos were injected with either RGDS, GRGESP, YIGSR peptides, PCS, or anti-fibronectin or anti-laminin antisera. A transverse section of each embryo was placed under the microscope, so that it appeared longitudinally in the field of view. With this constant 'length' of 320μm, and with the primitive streak situated centrally within the field, the distance between the mesoderm and the ectododerm at both the top and bottom of the field of view, and the distance between the mesoderm and endoderm, also at the top and bottom of the field of view, and the number of mesoderm cells seen to be touching the basement membrane of the ectoderm, were noted. Figure 10 shows the various measurements that were taken for each embryo. These values were then used for statistical comparison.

Results from a two-way analysis of variance (ANOVA) show that there are no significant differences at $p<0.05$ for the distance between the mesoderm cells, and the ectoderm, or for the distance between the mesoderm cells and the endoderm, for any of the treatments. At the same confidence limit, however, it appears that there is a significant difference between a number of the experimental groups, for the number of mesoderm cells attached to the basement membrane underlying the ectoderm.

Figure 9 - transverse section of a stage 5 chick embryo, examined using light microscopy, showing the presence of polystyrene beads between the ectoderm and the endoderm (arrows). (x341).

Figure 10 - transverse section of a stage 5 chick embryo, examined using light microscopy, showing the parameters that were measured, and used for statistical comparison. These included the distance between the mesoderm (m) and the ectoderm (ec), the distance between the mesoderm and the endoderm (en), the width of the embryo to one side of the primitive streak (ps), and the number of mesoderm cells migrating on the basement membrane (bm) of the ectoderm. (x293).

Figure 11a - stage 5 chick embryo (SEM) showing the area pellucida with the endoderm removed, to expose the migrating mesoderm beneath. (x122).
ao - Area opaca; ec - ectoderm; m - mesoderm; en - endoderm

Figure 11b - higher power magnification (SEM) of 11a, showing the partially removed endoderm (en), with the mesoderm (m), and the ectoderm beneath (ec). (x756)

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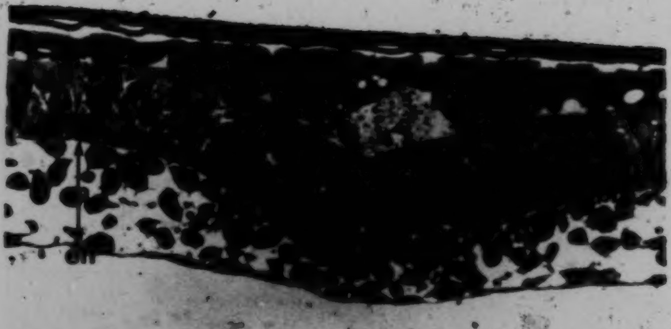

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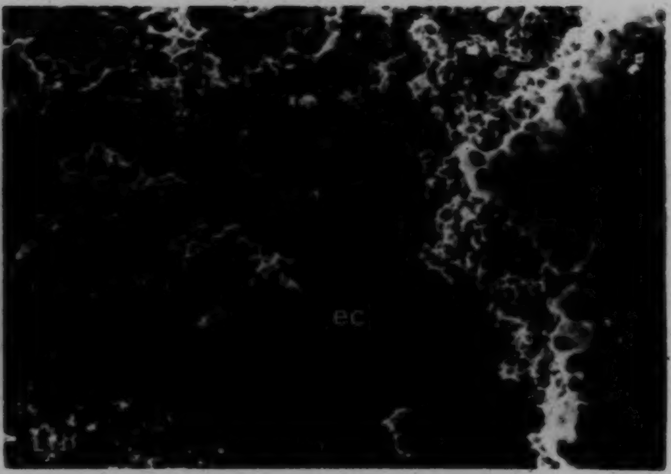
11a



11b

These significant differences are between a) GRGESP-treated embryos, and embryos exposed to anti-fibronectin antibodies, b) YIGSR-treated embryos, and those exposed to anti-fibronectin antibodies, c) YIGSR-treated embryos, and those exposed to the RGDS peptide (Table 1a, 1b). These results are not entirely unexpected, since the GRGESP peptide is a control peptide, and should, therefore, should not have an effect, and, if the mesoderm cells are utilizing the fibronectin in the basement membrane for migration, as previously hypothesized, the anti-fibronectin antibodies would block this interaction.

A more in depth comparison was carried out using a 2-tailed students t-test. At $p<0.05$, it was noted that there was also a significant decrease in the number of mesoderm cells attached to the basement membrane between d) RGDS-treated embryos in comparison to those exposed to GRGESP and also, e) embryos treated with anti-laminin antibodies, and those exposed to anti-fibronectin antibodies. In addition, and surprisingly, the t-test also detected a significant increase in the distance between the mesoderm cells and the endoderm, between embryos treated with anti-laminin antibodies, and those exposed to anti-fibronectin antibodies, and also between embryos treated with RGDS, and those exposed to anti-laminin antibodies. (Table 1a, 1b). This was a little unexpected, since it was presumed that the mesoderm cells would be using the basement membrane of the ectoderm on which to migrate.

Table 1a - summarizing the mean values, with standard deviation of embryos subjected to different treatments, and analysed at the light microscope level.

TREATMENT	n	dist. meso & ecto.	dist. meso. & endo.	no. cells on BM.
RGDS	16	3.0μm ± 5.6μm	5.2μm ± 7.5μm	9.3 ± 2.9
GRGESP	11	1.1μm ± 2.2μm	2.4μm ± 2.2μm	12.5 ± 2.7
YIGSR	8	1.0μm ± 2.3μm	2.5μm ± 3.2μm	14.1 ± 4.3
αLN	9	2.9μm ± 5.2μm	1.7μm ± 1.9μm	11.6 ± 3.0
αFN	12	3.2μm ± 5.1μm	4.1μm ± 4.9μm	7.18 ± 3.4

Table 1b - summarizing significant differences in a number of measured parameters, from embryos subjected to different treatments, and analysed at the light microscope level (2-way ANOVA**, and 2-tailed Students T-test*, $p<0.05$).

MEASURED PARAMETERS

TREATMENT	dist. meso. & ecto.	dist. meso. & endo.	no. cells on BM.
RGDS v GRGESP	-	-	*
GRGESP v αFN	-	-	*/**
αFN v YIGSR	-	-	*/**
YIGSR v RGDS	-	-	*/**
αLN v αFN	-	*	*
αLN v RGDS	-	*	-

The same peptides and antibodies were injected into embryos prepared for scanning electron microscopy. Initial experiments involved removing the endoderm from the dehydrated embryo, to expose the mesoderm beneath (Figure 11a, 11b). This proved inadequate, however, as it was not possible to view the interaction of the mesoderm cells with the ectoderm. Consequently, for further experiments, embryos were split transversely, across the primitive streak, at the mid-way point. This allowed an 'inside' view of the mesoderm between the two layers. Having taken a selection of scanning electron micrographs (SEM's) of mesoderm cells from regions close to the primitive streak, and from embryos subjected to different treatments, it was necessary to have a random method for choosing cells for analysis. Therefore, all those micrographs with one or more cell having an uninterrupted outline were taken, and used for a shape analysis. This involved tracing the uninterrupted outline of the cells, and being assigned a value between 0 and 1 (where 1 is equal to a perfect circle). An example of a tracing from Bioquant 3D, and its photographic counterpart can be seen in Figures 12a and 12b respectively. The range of values obtained from 'shape factor' for the various treatments can be seen in Table 2. Cells from RGDS-treated embryos (Figures 13, 14) appeared, generally, to have a smoother outline than cells from the control GRGESP, which have lamellipodia and filopodia (Figures 15, 16a, 16b). In some cases, RGDS-treated embryos displayed cells with a spiky appearance (Figure 17). YIGSR, anti-laminin antibody, and PCS treated embryos tended to show a similar morphology to those treated with GRGESP (Figures 18, 19), whilst those subjected to anti-fibronectin antiserum were more like RGDS-treated embryos (Figure 20).

A graphic representation of the data obtained from this analysis can be seen in Figure 21. In figure 21a, embryos were treated with fibronectin-associated peptides and antibodies. It can be seen that treatment with anti-fibronectin antibodies causes the cells to become more round than any other treatment. Embryos injected with the peptide RGDS are still rounder than those treated with the control peptide GRGESP. In figure 21b, embryos were treated with peptides and antibodies associated with laminin. In this figure, it appears that none of the laminin-associated treatments or the control peptide, appeared to have had a significant effect on the shape of the migrating mesoderm cells.

Statistical comparison of the data obtained from the shape factor analysis was carried out, using a one-way analysis of variance (ANOVA). At $p<0.05$,it was noted that there was a significant difference between GRGESP-treated embryos, and those treated with anti-fibronectin antibodies. A more in-depth analysis using the student T-test, revealed that, at $p<0.05$, there was a significant difference between the following treatments: a) RGDS-treated embryos, and those exposed to GRGESP, b) GRGESP-treated embryos, and those exposed to anti-laminin antibodies, c) GRGESP-treated embryos, and those exposed to YIGSR, d) embryos treated with anti-laminin antibodies, and those exposed to anti-fibronectin antibodies, and e) GRGESP-treated embryos, and those exposed to anti-fibronectin antibodies. The last result, which was also obtained from the ANOVA analysis, proved to be significant at $p<0.01$,when calculated using the students T-test (Table 3a, 3b).

Table 2 - showing the range of values obtained from 'shape factor' for experimental and control embryos.

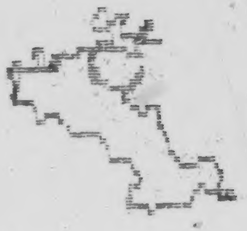
	NO. OF CELLS				
Limit	GRGESP	RGDS	YIGSR	αFN	αLN
<0.000	0	0	0	0	0
0.050	0	0	0	0	0
0.100	4	3	1	0	3
0.150	7	3	2	1	3
0.200	9	8	10	12	10
0.250	4	7	6	8	9
0.300	1	1	2	10	9
0.350	1	5	0	7	3
0.400	0	1	3	6	2
0.450	1	1	1	0	0
0.500	0	1	0	0	0
>0.500	0	0	1	0	0
	27	30	26	44	39

Figure 12a - cell tracing from the Bioquant 3D shape factor analysis (see figure 12b).

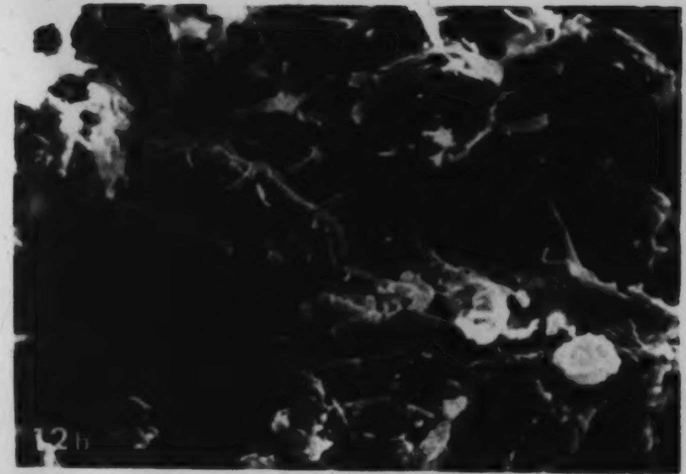
Figure 12b - SEM of a mesoderm cell (*) migrating on the basement membrane of the ectoderm corresponding to the cell tracing in figure 12a. Note that *in vivo*, the ectoderm is positioned dorsal to the mesoderm - this figure has been inverted. (x8850).

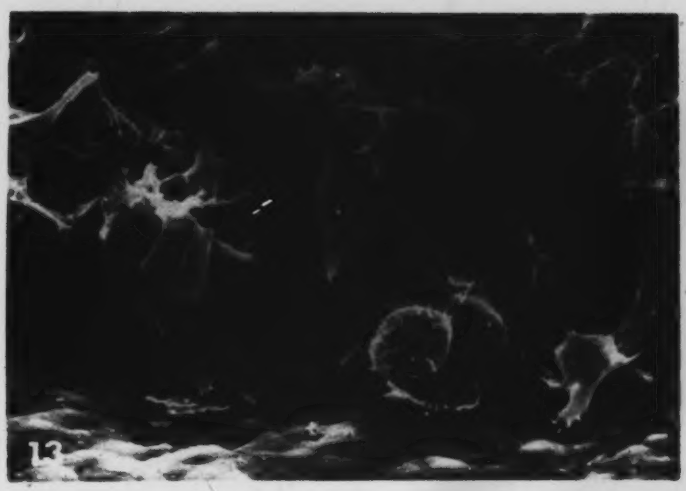
Figure 13 - SEM of a stage 5 chick embryo treated with the peptide RGDS. Note smooth appearance of the mesoderm cells. (x3930).

Figure 14 - SEM of a stage 5 chick embryo treated with the peptide RGDS. Note rounded morphology of the mesoderm cells. (x2895).



12a





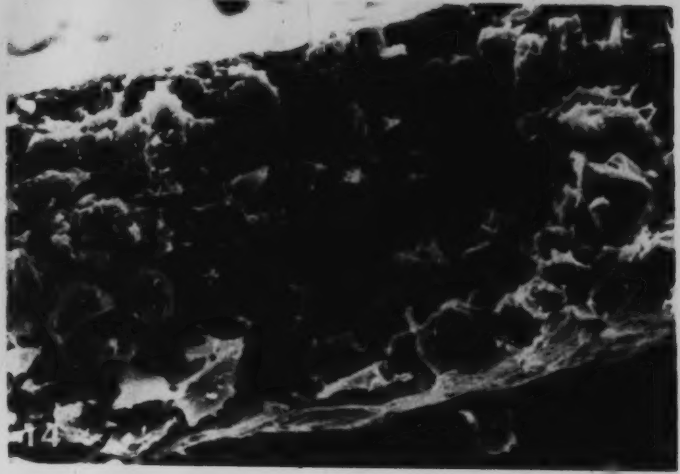
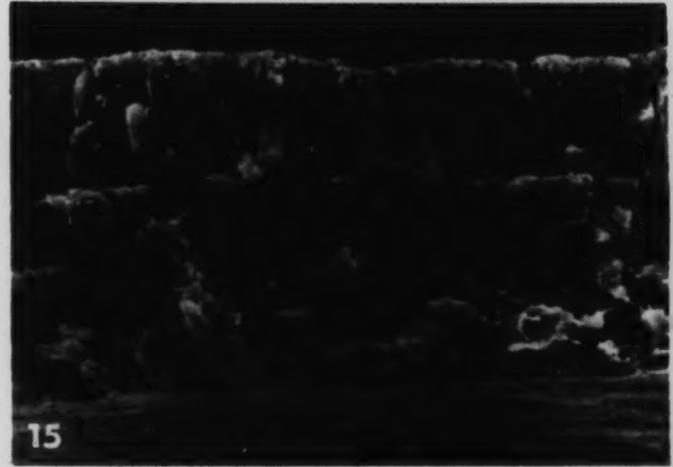


Figure 15 - SEM of a stage 5 chick embryo treated with the peptide GRGESP. (x2325).

Figure 16a - SEM of a stage 5 chick embryo treated with the peptide GRGESP. Note the numerous filopodia extending from the mesoderm cells towards the ectoderm (arrows). Figure has been inverted. (x9300).

Figure 16b - SEM of a stage 5 chick embryo treated with the peptide GRGESP. Note the lamellipodia extending from the mesoderm cell, along the basement membrane of the ectoderm (arrow). Figure has been inverted. (x5310).

Figure 17 - SEM of a stage 5 chick embryo treated with the peptide RGDS. Note the spiky appearance of the cells. (x2430).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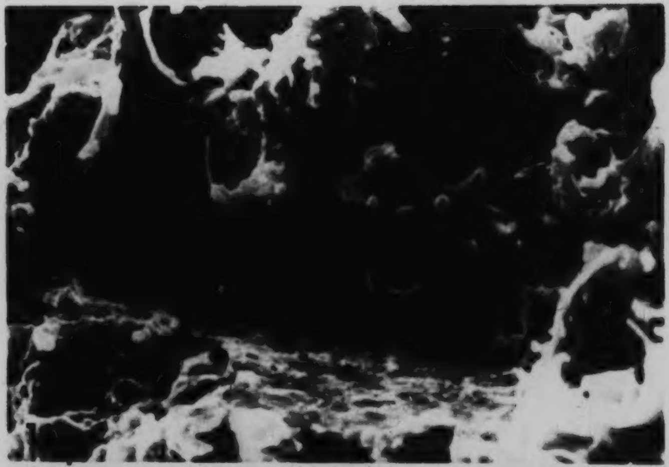

16b

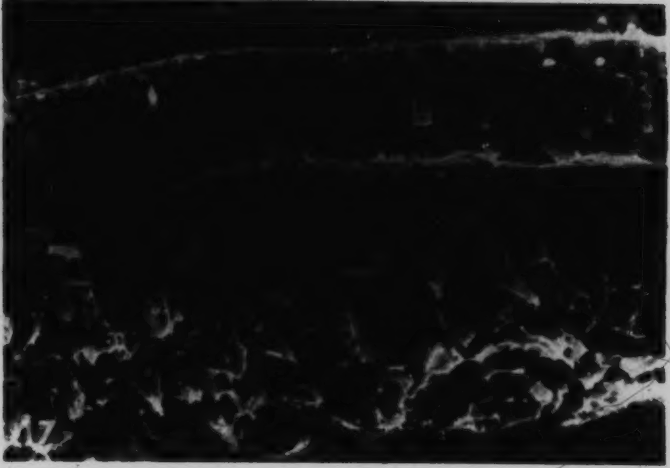
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Figure 18 - SEM of a stage 5 chick embryo treated with the peptide YIGSR. Note filopodia extending from the mesoderm towards the basement membrane of the ectoderm. Figure has been inverted. (x4860).

Figure 19 - SEM of a stage 5 chick embryo treated with anti-laminin antibodies. Note filopodial contacts between mesoderm cells. Figure has been inverted. (x3750).

Figure 20 - SEM of a stage 5 chick embryo treated with anti-fibronectin antibodies. Note rounder mesoderm cells, with smoother surfaces. (x5070).

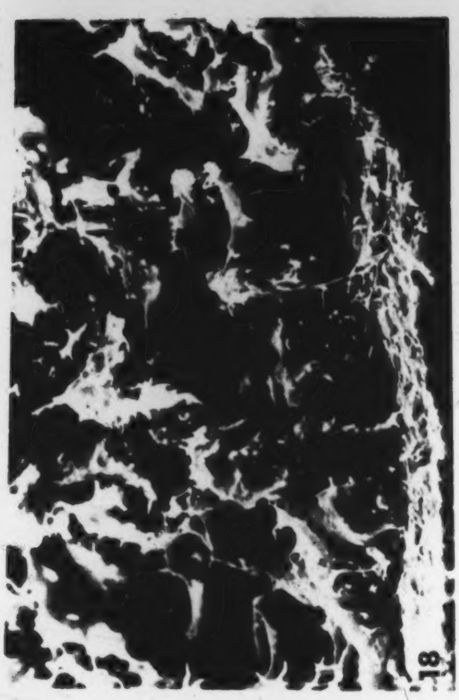

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Figure 21a - Histogram showing the relative roundness of mesoderm cells (as determined by shape factor) from embryos treated with RGD and RGE-containing peptides and anti-fibronectin antibodies. Note the distinct right shift (*ie.* increasingly more round) of cells from embryos treated with the peptide RGDS and those treated with anti-fibronectin antibodies.

SHAPE VARIATION AFTER TREATMENT WITH PEPTIDES OR ANTIBODIES

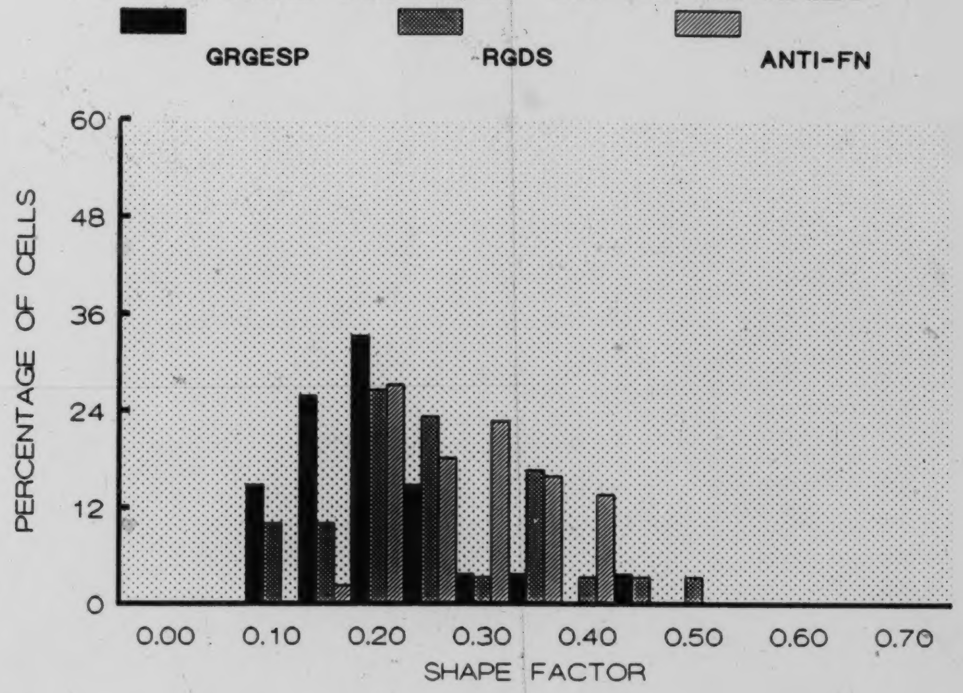


Figure 21b - Histogram showing the relative roundness of mesoderm cells (as determined by shape factor) from embryos treated with the YIGSR peptide, anti-laminin antibodies, or the control peptide, GRGESP. Note that there is little difference between the three treatments.

SHAPE VARIATION AFTER TREATMENT WITH PEPTIDES OR ANTIBODIES

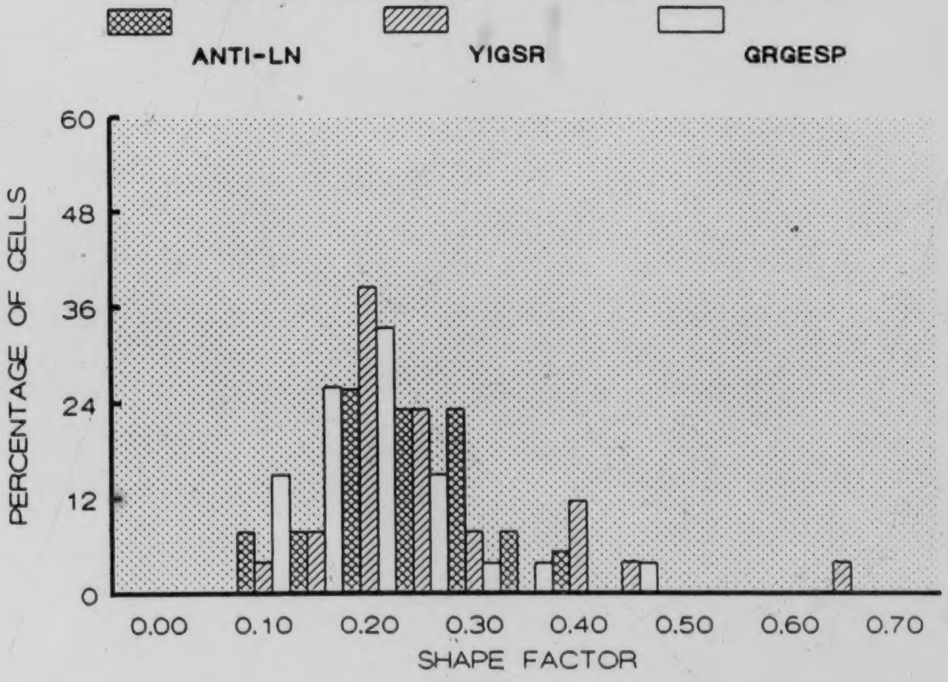


Table 3a - summarizing significant differences in the shape of cells obtained from subjected to different treatments, and analysed using scanning electron microscopy (2-way ANOVA**, and students T-test, 2-tailed*).

TREATMENT	sig. at $p<0.05$	sig. at $p<0.01$
RGDS v GRGESP	*	-
GRGESP v αLN	*	-
YIGSR v GRGESP	*	-
αFN v αLN	*	-
αFN v GRGESP	*/**	*/**

Table 3b - summarizing the mean and standard deviation values, with the sample size for experimental group, analysed at the scanning electron microscope level.

	x	sd	n
RGDS	0.227	0.102	30
GRGESP	0.169	0.081	27
YIGSR	0.237	0.117	26
PCS	0.233	0.080	41
αFN	0.260	0.074	44
αLN	0.218	0.076	39

IN VITRO EXPERIMENTS

When mesoderm cells are plated on uncoated glass with normal medium (199 medium plus 5% FBS (without fibronectin), plus gentamycin), they spread out from the explant, showing a fibroblastic morphology (Figure 22). If, however, mesoderm cells are plated on fibronectin, they grow as an epithelial sheet (Figure 23). Using these morphologically distinct traits as standards, mesoderm cells from stage four embryos (24 hrs; Hamburger & Hamilton, 1951) were cultured on a variety of substrata, and exposed to normal medium containing a number of different peptides and antibodies. Both the substrata and medium were thought to be associated with, or interfere with the normal migratory mechanisms of the mesoderm cells.

To prevent any exogenous fibronectin being utilized by the cells, and interfering with the results, normal fetal bovine serum was run through a gelatin-sepharose column (see Figure 4 for results of the Western blot). This ensured that any fibronectin in the serum bound to the gelatin, leaving a final eluent for experimental purposes, that was free of fibronectin.

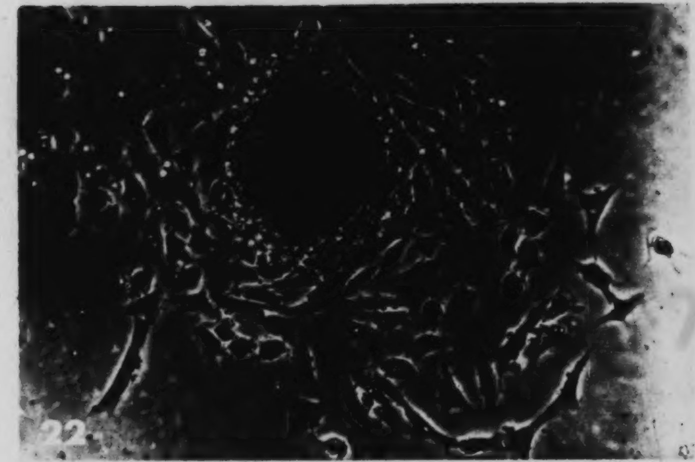
Experiments were carried out in batches, and were always accompanied by control experiments. The controls are described the first time they appear, but not thereafter.

Figure 22 - mesoderm cells cultured on uncoated glass with normal medium. Note the fibroblastic morphology. (x94).

Figure 23 - mesoderm cells cultured on fibronectin-coated glass with normal medium. Note the epithelial morphology. (x94).

Figure 24 - mesoderm cells cultured on fibronectin-coated glass with RGDS-containing medium at a final concentration of 10^{-3}M. Note epithelial morphology remains unaffected. (x94).

Figure 25a - mesoderm cells cultured on fibronectin-coated glass with RGDS-containing medium at a final concentration of 5×10^{-3}M. Note the mixed morphology of cell types, including epithelial sheets, single rounded cells, and individual spread cells. (x94).



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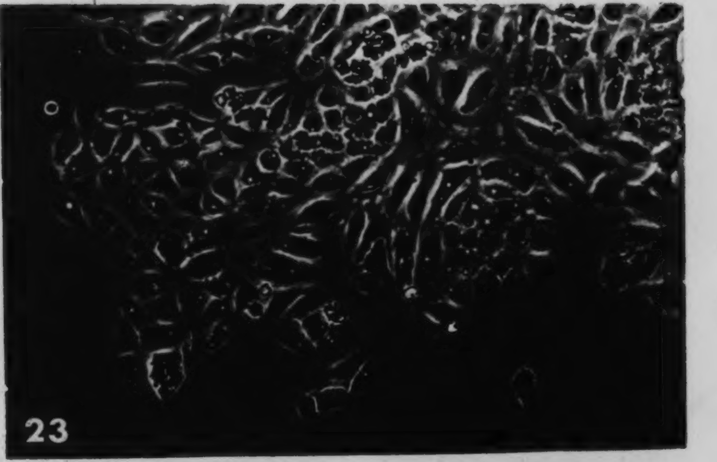

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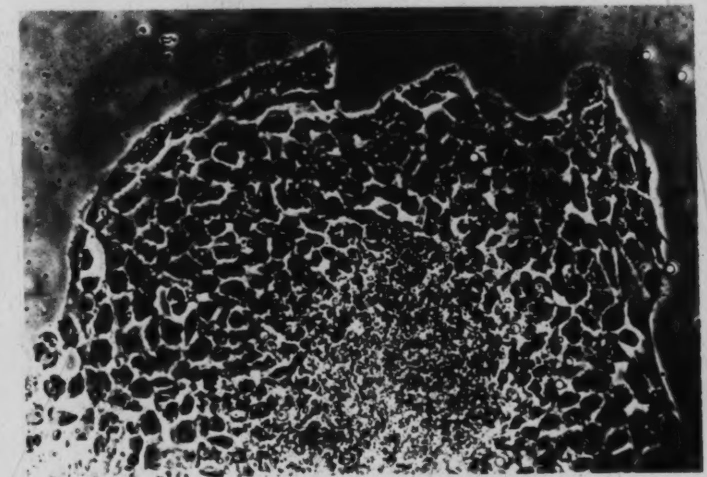
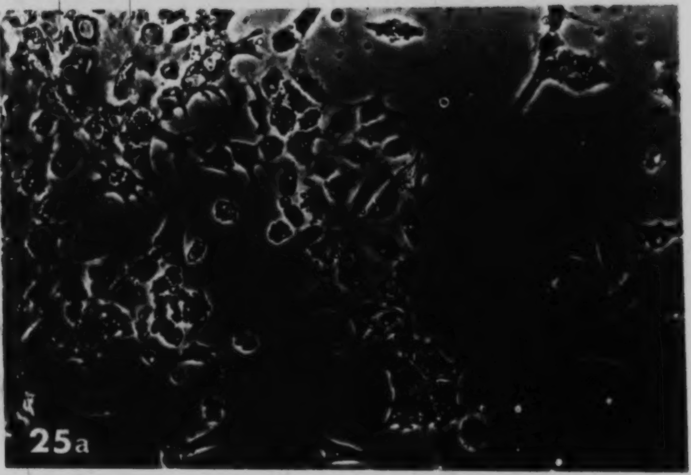




25a

1) a) On uncoated glass with normal medium (n=87)

   b) On fibronectin (FN)-coated glass with normal medium (n=58)

   c) On FN-coated glass with RGDS-containing medium (n=36)

   d) On uncoated glass with RGDS-containing medium (n=38)

c) Initial experiments were performed with a range of RGDS dilutions. At a dilution of 10^{-3}M, the mesoderm clumps on fibronectin appeared not to be affected by the peptide, and grew on the fibronectin as epithelial sheets (Figure 24). At 5 x 10^{-3}M, mesoderm cells on fibronectin displayed mixed morphologies. Some explants were attached, whilst others remained detached. Those that had spread, consisted of epithelial sheets, individual spread cells, and both single and groups of rounded cells (Figures 25a, 25b). Finally, at a dilution of 10^{-2}M, in most cases, there was no growth out from the mesoderm clumps on fibronectin, and occasionally, some of the cells appeared to have disintegrated (Figure 26). Subsequent experiments, therefore, were performed at a dilution of 5 x 10^{-3}M.

d) Mesoderm plated on uncoated glass and exposed to medium containing the RGDS peptide, usually attached to the glass, but appeared not to spread, and remained as a clump (Figure 27). Any cells that were detached from the main explant, displayed a rounded morphology.

Figure 25b - mesoderm cells cultured on fibronectin-coated glass with RGDS-containing medium at a final concentration of 5×10^{-3}M. Note the mixed morphology of cell types, including epithelial sheets, single rounded cells, and individual spread cells. (x94).

Figure 26 - mesoderm cells cultured on fibronectin-coated glass with RGDS-containing medium at a final concentration of 10^{-2}M. Note that there is no outgrowth from the clump of mesoderm. (x188).

Figure 27 - mesoderm cells cultured on uncoated glass with RGDS-containing medium (5×10^{-3}M). Note that there is no outgrowth from the clumps of mesoderm, and any cells that are detached from the main explant show a rounded morphology. (x94).

26

27

In this set of experiments, the results were quantitated, allowing a more objective rather than subjective analysis for comparison with other experiments. With mesoderm cultures on glass exposed to normal medium, the number of single, spread cells were counted. For mesoderm cultured on glass with RGDS-containing medium, rounded cells were counted. Since mesoderm on fibronectin grows as an epithelial sheet, more for comparative reasons, the number of cells per sheet, that fell within the grid, were noted. Finally, for cultures where mesoderm was grown on fibronectin in the presence of RGDS, individual cells, both rounded and spread, were counted, including those forming epithelial sheets.

Figure 28 gives a graphic representation of the variation in cell appearance throughout a number of experimental manipulations. Figure 28a, b, & c, show standard morphologies for their particular treatments. In every case, mesoderm cells cultured on uncoated glass coverslips with normal medium spread out from the main mass, and displayed a fibroblastic morphology (Figure 28a). The same cells grown on uncoated glass coverslips, but with medium containing RGDS, did not grow, and displayed, 100% of the time, a rounded morphology (Figure 28b). Mesoderm cells grown on fibronectin-coated coverslips with normal medium, spread outwards from the main mass, but displayed an epithelial morphology (Figure 28c). However, if mesoderm cells were cultured on fibronectin-coated coverslips with medium containing RGDS, they displayed a mixed morphology, with slightly more rounded cells per culture, than spread cells (Figure 28d).

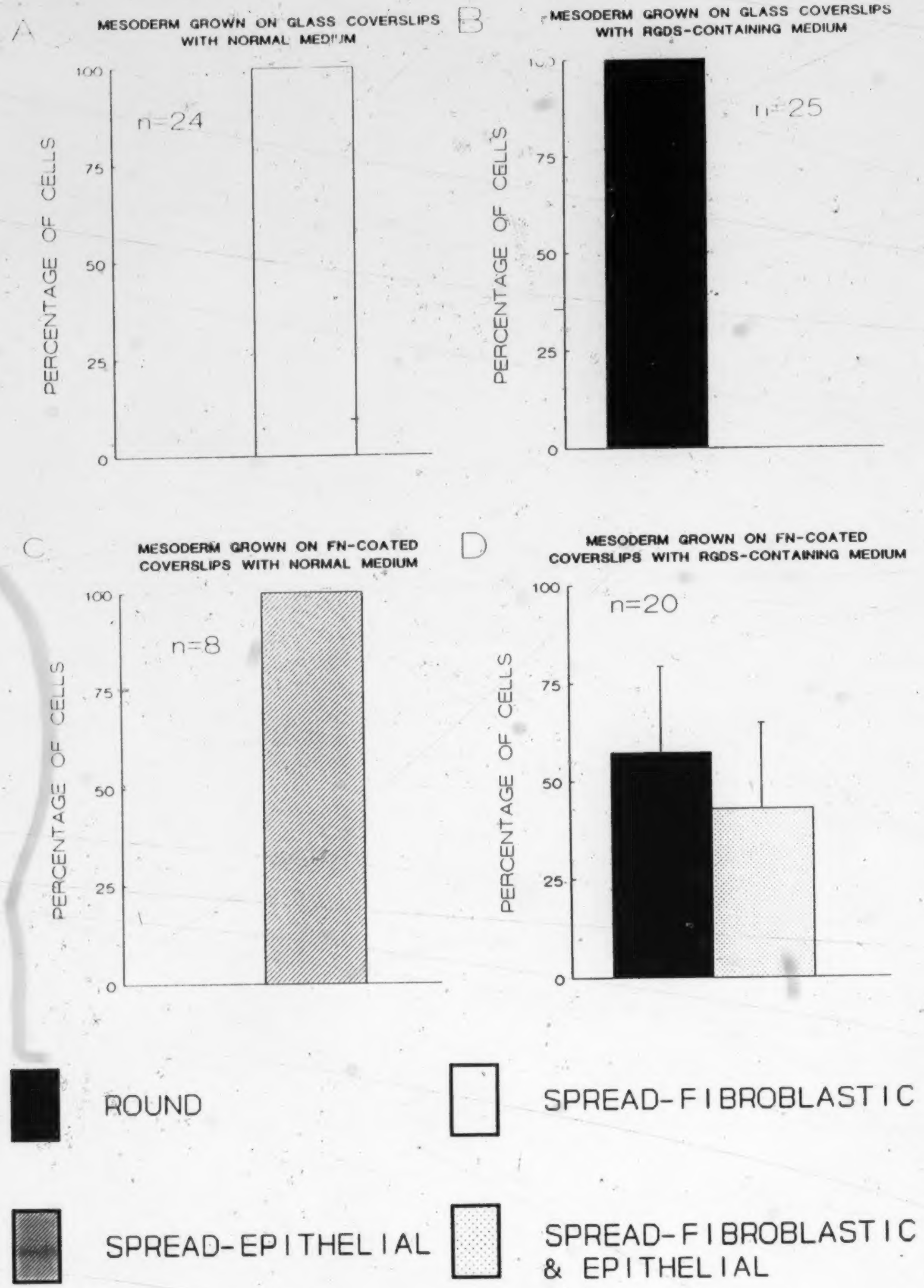
Figure 28 - graphic representation of the variation in cell appearance throughout a number of experimental manipulations (see experiment 1 in the results section).

In 28a, mesoderm cells cultured on uncoated glass with normal medium constantly displayed a fibroblastic morphology.

In 28b, mesoderm cells cultured on uncoated glass with RGDS-containing medium, constantly displayed a rounded morphology.

In 28c, mesoderm cells cultured on fibronectin-coated glass with normal medium, constantly displayed an epithelial morphology.

In 28d, mesoderm cells cultured on fibronectin-coated glass with RGDS-containing medium, displayed both rounded and spread (fibroblastic and epithelial) morphologies. In the majority of cases, cultures displayed more rounded cells, than spread cells.



A MESODERM GROWN ON GLASS COVERSLIPS WITH NORMAL MEDIUM

B MESODERM GROWN ON GLASS COVERSLIPS WITH RGDS-CONTAINING MEDIUM

C MESODERM GROWN ON FN-COATED COVERSLIPS WITH NORMAL MEDIUM

D MESODERM GROWN ON FN-COATED COVERSLIPS WITH RGDS-CONTAINING MEDIUM

Experiments were also performed where mesoderm cells were grown on fibronectin-coated coverslips with normal medium, left for 24 hours, and then the medium was replaced with medium containing RGDS. Cells were observed to change from the usual epithelial sheets, expected from mesoderm grown on fibronectin, to a mixture of individual spread cells, epithelial sheets, and rounded cells, the expected result with the addition of RGDS (Figures 29a, 29b). Further experiments were performed, of a similar nature. In the first, mesoderm cells were grown on fibronectin-coated coverslips with medium containing RGDS, and then replaced 24 hours later with normal medium. The opposite effect was seen from the previous experiment described. Cells were seen to change from an assortment of morphologies, to epithelial sheets (Figure 30). The final experiment involved culturing mesoderm cells on glass coverslips (untreated) with medium containing RGDS, which was replaced 24 hours later, with normal medium. Mesoderm grown in RGDS on glass, showed no signs of spreading, however, after the medium was replaced, the cells started to grow out from the mass as individual cells (Figure 30).

Each one of these experiments shows that the treatment is reversible, and is in no apparent way, deleterious to the cells.

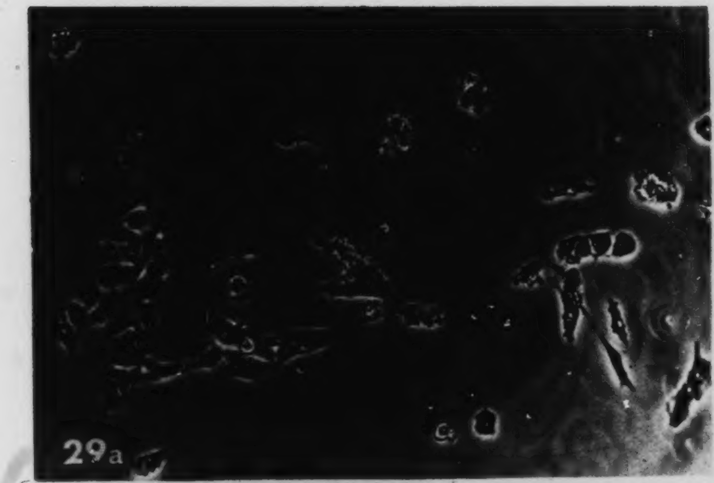
2) a) On uncoated glass with normal medium (n=87)
   b) On laminin (LN)-coated glass with normal medium (n=18)
   c) On LN-coated glass with YIGSR-containing medium (n=28)
   d) On uncoated glass with YIGSR-containing medium (n=40)

Figure 29a - mesoderm cells cultured on fibronectin-coated glass with normal medium, and left for 24 hours. The medium was then replaced with medium containing the peptide RGDS (5×10^{-3}M). Note that the mesoderm cells have altered their morphology from epithelial to an assortment of rounded, fibroblastic and epithelial types. (x94).

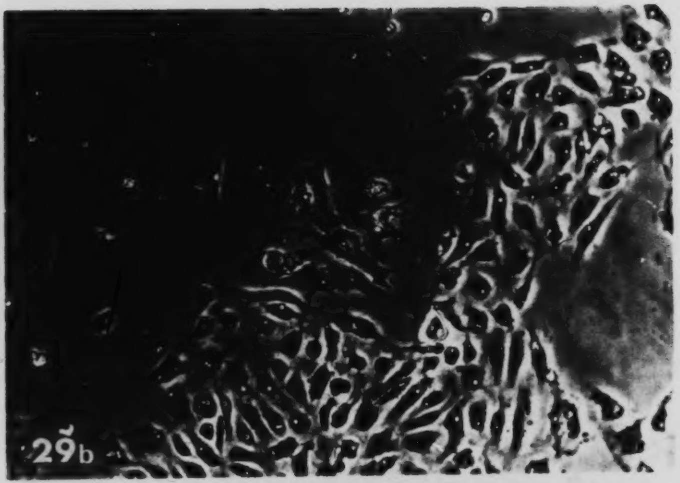
Figure 29b - mesoderm cells cultured on fibronectin-coated glass with normal medium, and left for 24 hours. The medium was then replaced with medium containing the peptide RGDS (5×10^{-3}M). Note the mesoderm cells moving individually away from the epithelial sheets, displaying a fibroblastic morphology. (x94).

Figure 30 - mesoderm cells cultured on fibronectin-coated glass with medium containing the peptide RGDS. Normal medium was then substituted 24 hours later. Note that the mesoderm cells changed from an assortment of morphologies, to epithelial sheets. (x94).

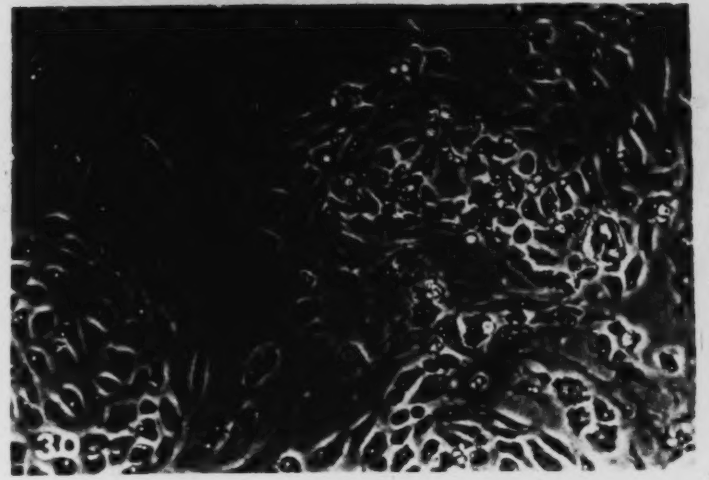
Figure 31 - mesoderm cells cultured on untreated glass with medium containing the peptide RGDS. Normal medium was then substituted 24 hours later. Mesoderm cells grown on untreated glass with RGDS do not spread. Note that the cells have started to migrate away from the main mass, displaying a fibroblastic morphology. (x94).



29a



29b



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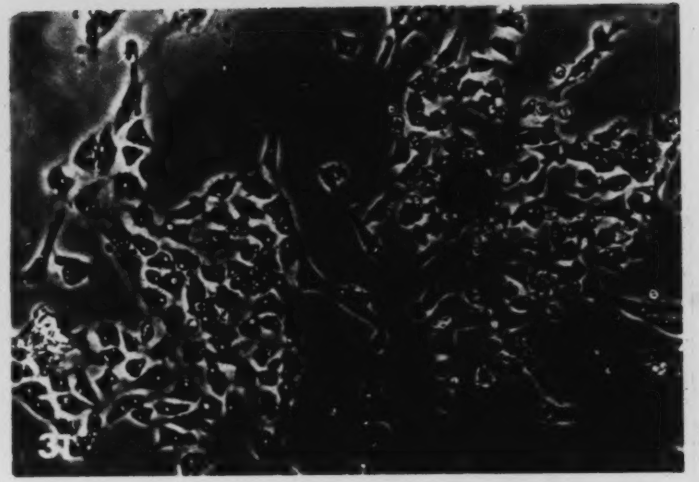

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b) Mesoderm cells grown on laminin, and exposed to normal medium, tended to show a similar morphology to those cultured on glass with normal medium *ie*. individual spread cells (Figure 34).

c) Initial experiments using YIGSR in the medium were performed with a range of YIGSR dilutions. At a dilution of 10^{-3}M, the mesoderm clumps appeared to have attached to the laminin, but there was no apparent outwards growth from the clump (Figure 32). At a dilution of 5 x 10^{-3}M, a few of the mesoderm clumps had attached to the laminin, although the majority had not (Figure 33). None of the cultures, however, showed any signs of spreading. Finally, at a dilution of 10^{-2}M, there was no attachment or spreading, and in some instances, some of the cells appeared to have disintegrated. Subsequent experiments used a dilution of 5 x 10^{-3}M.

d) Mesoderm cultured on glass with YIGSR-containing medium tended to remain in a clump, unattached to the glass, and surrounded by a mass of rounded cells. None of the cells appeared to have spread (Figure 35).

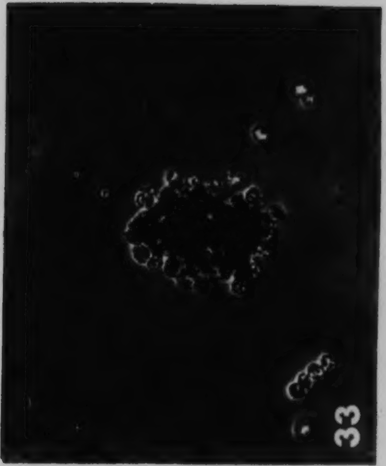
Experiments were also performed, where mesoderm cells were grown on laminin-coated coverslips with YIGSR-containing medium, left for 24 hours, and then the medium was replaced with normal medium. Examination of the cultures 24 hours later revealed that at dilutions of 10^{-3}M, and 5 x 10^{-3}M, clumps of mesoderm which

Figure 32 - mesoderm cells cultured on laminin-coated glass with medium containing the peptide YIGSR at a final concentration of 10^{-3}M. Note that there is no outgrowth from the mesoderm mass. (x109).

Figure 33 - mesoderm cells cultured on laminin-coated glass with medium containing the peptide YIGSR at a final concentration of 5×10^{-3}M. Note that there is no outgrowth from the mesoderm mass. (x109).

Figure 34 - mesoderm cells cultured on laminin-coated glass with normal medium. Note that the cells tend to show a similar morphology to those cultured on glass with normal medium (Fig.22) *ie*. fibroblastic cells. (x94).

Figure 35 - mesoderm cells cultured on untreated glass with medium containing the peptide YIGSR. Note that the cells have not grown, and tend to remain rounded. (x94).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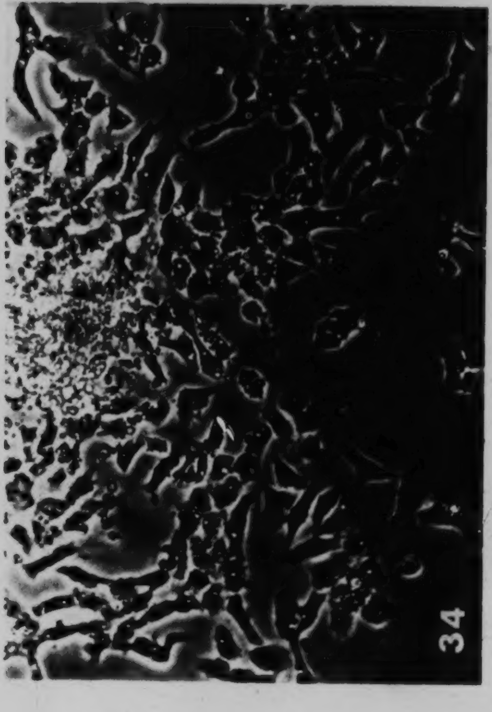
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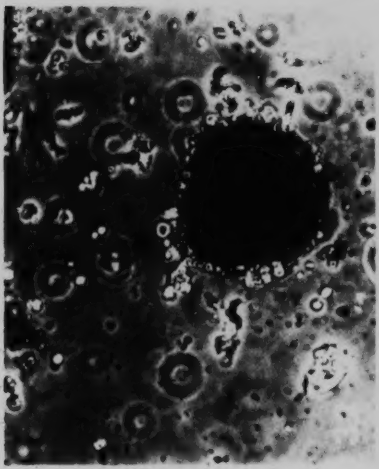


had previously shown no growth, displayed numerous cells spreading outwards from the mesoderm mass (Figures 36, 37). At a dilution of 10^{-2}M, cell spreading was also observed, but not to the same degree (Figure 38). Some of the cultures at this dilution also showed signs of disintegration. These results indicate that the treatment is reversible, at least at the two lower concentrations used, and was therefore, in no apparent way, deleterious to the cells.

Further quantitation of the results was not attempted, since it was not always possible to provide a meaningful value, and, since each experiment was testing a different factor, with different results, comparison between the variousgroups of data, would not have been possible.

3) a) On uncoated glass with GRGESP-containing medium (n=6)
   b) On FN-coated glass with GRGESP-containing medium (n=6)
   c) On uncoated glass with rabbit IgG-containing medium (n=8)
   d) On FN-coated glass with rabbit IgG-containing medium (n=12)
   e) On Ln-coated glass with rabbit IgG-containing medium (n=6)

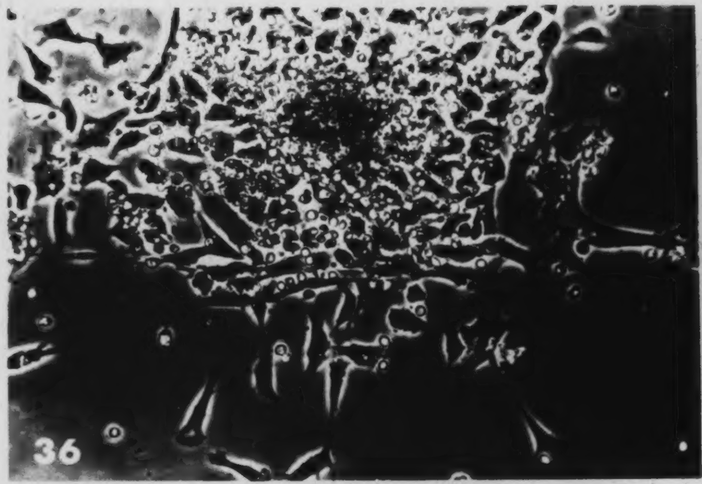
a) Mesoderm cells that were grown on uncoated coverslips and exposed to the GRGESP peptide, were seen to grow out from the main mass, and towards the periphery of the mass, spread as individual cells. This is analogous to the appearance that mesoderm displays when it is cultured on glass coverslips with normal medium.

Figure 36 - mesoderm cells cultured on laminin-coated glass with medium containing the peptide YIGSR (10^{-3}M). Normal medium was substituted 24 hours later. Note that the mesoderm cells have grown outwards from the main mass displaying a fibroblastic morphology. (x94).

Figure 37 - mesoderm cells cultured on laminin-coated glass with medium containing the peptide YIGSR (5×10^{-3}M). Normal medium was substituted 24 hours later. Note that the mesoderm cells have grown outwards from the main mass. (x94).

Figure 38 - mesoderm cells cultured on laminin-coated glass with medium containing the peptide YIGSR (10^{-2}M). Normal medium was substituted 24 hours later. Note that the mesoderm cells have grown outwards from the main mass, but not to the same degree as those cultured in medium containing lower concentration of YIGSR. Note also, the yolk droplets which are seen as vesicle-like structures within the cells. (x94).

Figure 39 - mesoderm cells cultured on fibronectin-coated glass with medium containing the peptide GRGESP. Note that the cells have grown as an epithelial sheet, indicating that the peptide has no effect on the growth of the cells. (x94).



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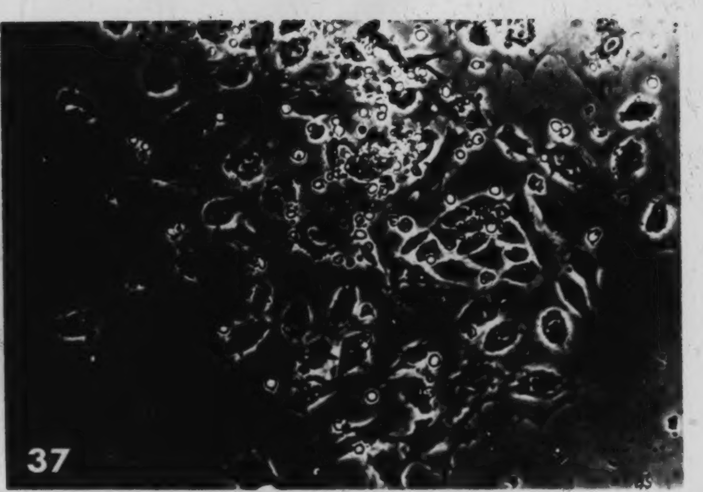

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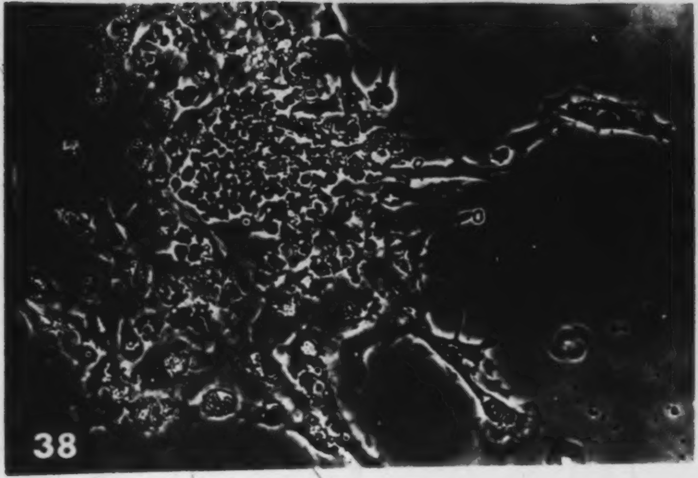

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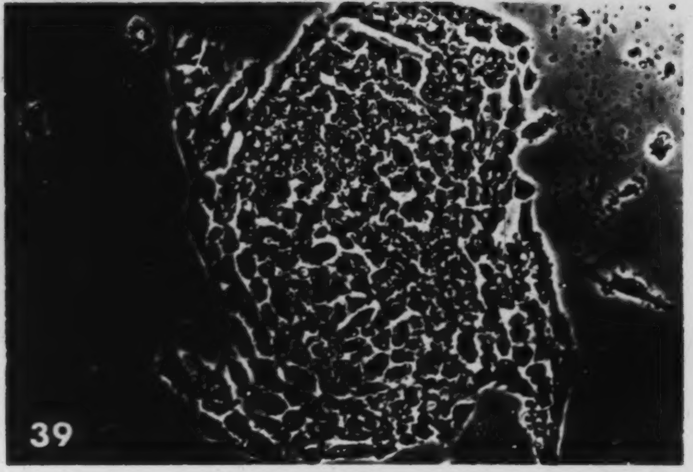

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b) When mesoderm cells were grown in the presence of GRGESP, but the coverslip was coated with fibronectin, the cells grew as an epithelial sheet, which is the same morphology observed when the cells are grown on fibronectin in the presence of normal medium (Figure 39). The GRGESP peptide therefore acts as a control, since it has no apparent effect on the cultured cells, even when the substrate in each experiment is different.

c) On uncoated glass with normal medium containing rabbit IgG, the mesoderm cells grew with a morphology similar to that of cells grown on glass with normal medium (Figure 40).

d) On fibronectin-coated glass with rabbit IgG in the medium, the cells grew as epithelial sheets (Figure 41).

e) On laminin-coated glass with IgG, the cells grew and shared the same kind of morphology as those grown on laminin with normal medium (Figure 42).

It is evident from this batch of experiments, that neither the GRGESP peptide, nor rabbit IgG, which were used as controls for experiments in which the RGDS peptide, and antibodies were used, respectively, had any effect on the cells that were grown in medium containing either of these two factors.

4) a) On uncoated glass with normal medium (n=87)
   b) On LN-coated glass with normal medium (n=40)
   c) On uncoated glass with RGDS-containing medium (n=38)
   d) On LN-coated glass with RGDS-containing medium (n=8)

When this study first began, it appeared that the RGDS sequence was not present in laminin (Martin & Timpl, 1987). It has since been demonstrated, however, that laminin does possess the RGDS sequence, in the short arm of the A chain (Grant *et al.*, 1989). This discovery prompted this particular experiment.

d) In most instances, the mesoderm clumps cultured on a laminin substrate, with RGDS in the medium, appeared to have attached to the substratum, but no spreading had occurred. Clumps were surrounded by a mass of rounded cells (Figure 43).

5) a) On uncoated glass with normal medium (n=87)
   b) On FN-coated glass with normal medium (n=58)
   c) On uncoated glass with anti-fibronectin antiserum (n=8)
   d) On FN-coated glass with anti-fibronectin antiserum (n=6)

c) & d) Cells cultured on glass, or fibronectin, and treated with anti-fibronectin antiserum, remained in their original clump, and showed only a minimal amount of

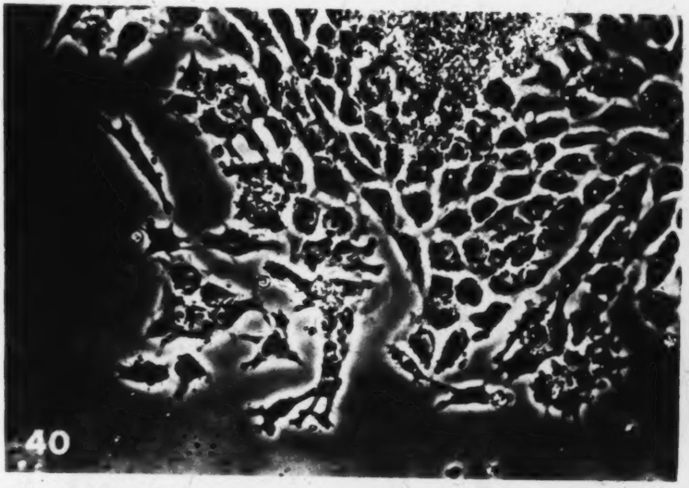
Figure 40 - mesoderm cells cultured on untreated glass with medium containing rabbit IgG. Note that the cells display the same morphology as those cultured on untreated glass with normal medium, (Fig.22) *ie.* fibroblastic, indicating that the rabbit IgG has no effect on the cells. (x94).

Figure 41 - mesoderm cells cultured on fibronectin-coated glass with medium containing rabbit IgG. Note that the cells display the same morphology as those cultured on fibronectin-coated glass with normal medium (Fig.23) *ie.* epithelial, indicating that the rabbit IgG has no effect on the cells. (94).

Figure 42 - mesoderm cells cultured on laminin-coated glass with medium containing rabbit IgG. Note that the cells display the same morphology as those cultured on laminin-coated glass with normal medium (Fig.34) *ie.* fibroblastic, indicating that the rabbit IgG has no effect on the cells. (94).

Figure 43 - mesoderm cells cultured on laminin-coated glass with medium containing the peptide RGDS. Note that there is no outwards growth from the main mass. (x109).

Figure 44 - mesoderm cells cultured on untreated glass or fibronectin-coated glass, and treated with medium containing anti-fibronectin antiserum. Note that there is no growth from the main mesoderm mass. (x109).



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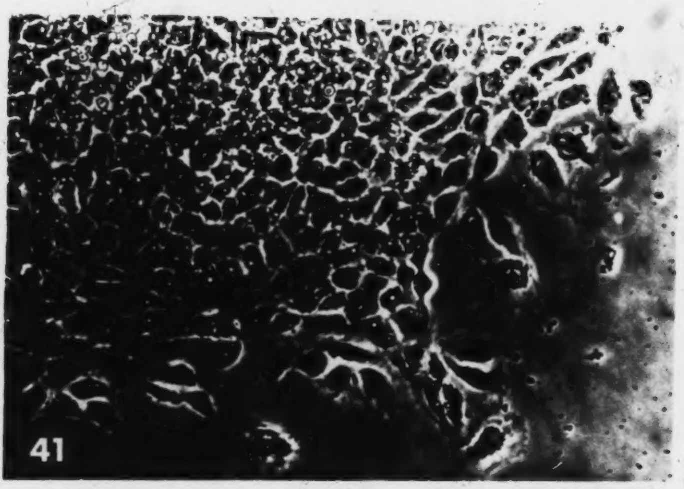

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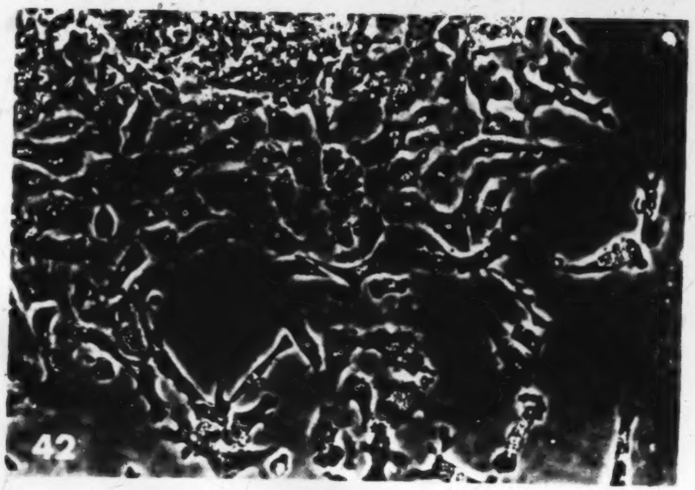

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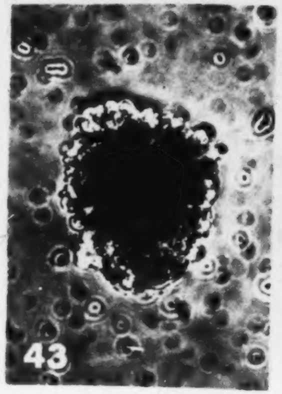

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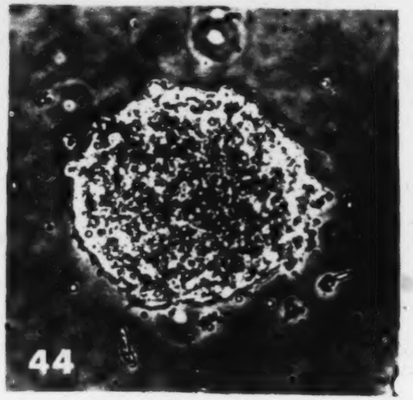

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blebbing. There was no evidence of any spreading having occurred (Figure 44).

6) a) On uncoated glass with normal medium (n=87)
   b) On LN-coated glass with normal medium (n=40)
   c) On uncoated glass with anti-laminin antiserum (n=2)
   d) On LN-coated glass with anti-laminin antiserum (n=8)

c) & d) Cells cultured on glass, or laminin, and treated with anti-laminin antiserum, remained as clumps, and did not spread, although some blebbing was observed (Figure 45).

7) a) On uncoated glass with normal medium (n=87)
   b) On uncoated glass with anti-fibronectin receptor antibody (n=10)
   c) On FN-coated glass with anti-fibronectin receptor antibody (n=12)

Initial experiments were performed with a range of anti-fibronectin receptor antibody dilutions. At a dilution of 1:10 with normal medium on a fibronectin substrate, the mesoderm cells were unable to attach to their substratum. At a dilution of 1:50, some clumps had attached, and some had not, but there appeared not to be any spreading. Finally, at 1:100, most, but not all clumps had attached, but there was still no spreading. Subsequent experiments used a dilution of 1:40, which is the standard dilution for other antibodies used in this study.

b) Mesoderm cultured on glass, and treated with anti-fibronectin receptor antibodies, was generally attached to the glass, but showed no evidence of spreading. In some instances, a few rounded cells could be observed surrounding the main mesoderm mass (Figures 46).

c) On fibronectin-treated glass, and subjected to the same antibody, mesoderm cells displayed varied morphologies (Figure 47), similar to those shown by the same cells exposed to RGDS .

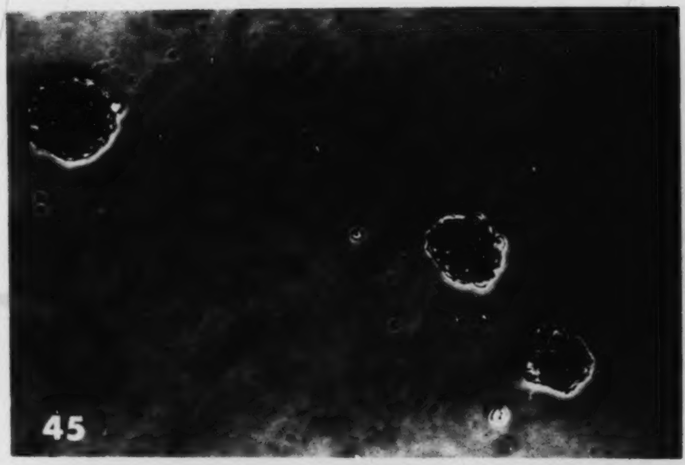
Towards the end of the experiments, it was observed that, in contrast to mesoderm cells, endodermal tissue was not affected by the presence of the anti-fibronectin receptor antibody. It was decided, therefore, to culture endoderm under the same conditions as the mesoderm in this particular batch of experiments. Interestingly, the anti-fibronectin receptor antibody had no effect on the growth of the endoderm on either glass, or fibronectin substrata. The endoderm grew as an epithelial sheet, which conforms with its usual morphology (Figures 48).

Figure 45 - mesoderm cells cultured on laminin-coated glass and treated with anti-laminin antiserum. Note that there was no outgrowth from the main mesoderm masses. Mesoderm cells cultured on untreated glass and exposed to anti-laminin antiserum also display the same morphology (x94).

Figure 46 - mesoderm cells cultured on untreated glass with medium containing anti-fibronectin receptor antibodies. Note that there was no outgrowth from the main mesoderm mass. (x94).

Figure 47 - mesoderm cells cultured on fibronectin-coated glass with medium containing anti-fibronectin receptor antibodies. Note that the morphology of the cells is similar to those cultured on fibronectin and treated with RGDS (Fig.25a, 25b) *ie.* mixed morphologies. (x94).

Figure 48 - endoderm tissue cultured on fibronectin-coated glass with medium containing anti-fibronectin receptor antibodies. Note that the endoderm retains its usual epithelial morphology. Mesoderm cultured on untreated glass with medium containing anti-fibronectin receptor antibodies also display the same morphology (x94).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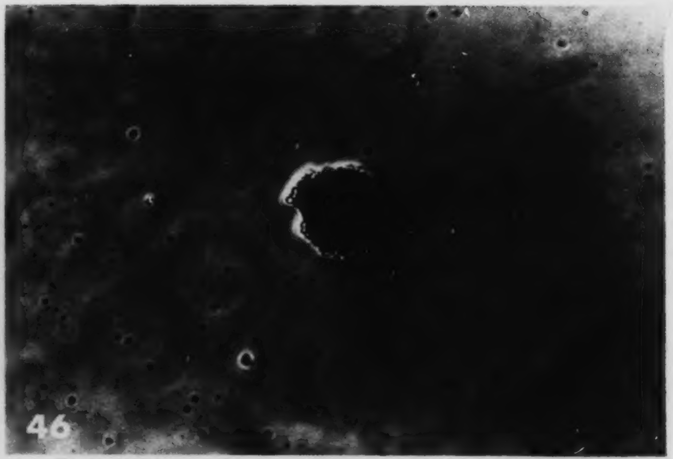
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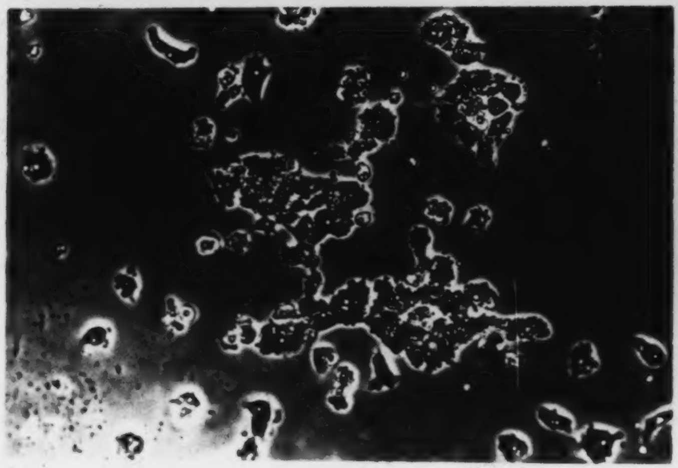

4

DISCUSSION

During the early stages of chick development, epiblast cells move medially towards the primitive streak, through which they invaginate, and then move laterally between the developing ectoderm and endoderm. These mesoderm cells interact with one another, in addition to the ectoderm, which is underlaid by a basement membrane. They also associate with the endoderm, but to a lesser extent. It is known that the majority of basement membranes found associated with cells, contain, as one of their major constituents, the large glycoprotein, fibronectin (Timpl & Dziadek, 1986). It is also known that fibronectin has a number of binding regions, one of which is the cell-binding tetrapeptide Arg-Gly-Asp-Ser (RGDS; Pierschbacher & Ruoslahti, 1984a; Ruoslahti & Pierschbacher, 1987).

Synthetic peptides containing this sequence have previously been microinjected into gastrulating amphibian embryos (Boucaut *et al.*, 1984b). Such embryos developed abnormally, and no migrating cells were found on the blastocoel roof. However, rounded cells were found to be dispersed along the blastocoel floor, in contact with endodermal cells. *In vitro*, avian neural crest cells exposed to the same peptide, would not migrate on a fibronectin substratum, and would detach and round up, if the peptide was added after the cells had begun to migrate (Boucaut *et al.*, 1984b; Perris & Bronner-Fraser, 1989). Injection of the peptide *in vivo*, prevented the forward migration of the neural crest cells along their normal pathway (Boucaut *et al.*, 1984b).

The primary aim of this project, was to determine whether the fibronectin cell-binding tetrapeptide, RGDS, would have any effect on the migrating mesoderm cells of the gastrulating chick embryo. Both fibronectin (Critchley *et al.*, 1979; Mitrani & Faberov, 1982; Sanders, 1982), and to a lesser extent, laminin, (Mitrani, 1982; Bortier *et al.*, 1989), have been shown to be present at this stage of development, and, although fibronectin is known to influence the migration of cells, *in vitro* (Sanders, 1980), it is still uncertain as to the role of fibronectin in the embryo at this stage of development. For this reason, it was decided to inject the minimum cell-binding recognition site (RGDS) into the stage 3 - 4 chick embryo, with the hypothesis that, if fibronectin was indeed involved in migration, the peptide would competitively inhibit the attachment of the mesoderm cells to the fibronectin in the basement membrane, and lead to a possible alteration in the cells' morphology. As previously mentioned, laminin is also present at this time, although not to the same extent, and so it was also decided to microinject the laminin cell-binding pentapeptide, YIGSR, into the chick embryo, to determine whether this would have any effect on the migrating cells. To complement these injections, anti-fibronectin, and anti-laminin antibodies were injected, since these will completely block their respective glycoproteins, and, if fibronectin and/or laminin are involved in migration, would be expected to show similar results to the peptides. Finally, the peptide GRGESP, and PCS were injected to act as controls, for RGDS and YIGSR.

Analysis of the statistics obtained from the light microscope studies (two-way analysis of variance - ANOVA) did not detect any significant difference between the distance between the mesoderm and the ectoderm, or between the mesoderm and endoderm, for any of the treatments. Further investigation, however, using a Student's t-test, revealed a significant difference at $p<0.05$,for the distance between the mesoderm and endoderm, between embryos treated with anti-laminin antibodies, and those exposed to anti-fibronectin antibodies, and also between embryos treated with anti-laminin antibodies, and those exposed to the peptide RGDS. These results were a little unexpected, since the dorsal surface of the endoderm does not possess a basement membrane to the same extent as the ventral surface of the ectoderm, and also appears to be poor in fibronectin (Sanders, 1980). There could be a number of possibilities to explain these results. Firstly, the data obtained for the distance between the mesoderm and ectoderm, for embryos treated with anti-fibronectin antibodies, and also with the peptide RGDS, contain a number of values higher than the average value. In general, such values were not consistent, and may therefore, not adequately represent this particular parameter. Secondly, embryos treated with the peptide RGDS, and those treated with anti-fibronectin antibodies, have a greater sample size than embryos treated with anti-laminin antibodies, and this may also affect the results.

However, it appeared that there was a significant difference in the number of mesoderm cells attached to the basement membrane both by the ANOVA and the t-test. Embryos treated with anti-fibronectin antibodies differed significantly for this particular parameter, from embryos treated with either the GRGESP, or the YIGSR peptides. GRGESP is a control peptide, which does not have any effect on the adhesion of the cells. Anti-fibronectin antibodies, however, will block the entire fibronectin molecule, and prevent the mesoderm cells from attaching to it. The YIGSR peptide, although it binds to the laminin receptor present on the mesoderm cells, does not appear to inhibit their attachment to the basement membrane to the same extent as the anti-fibronectin antibodies. Similarly, there was a significant difference between YIGSR and RGDS-treated embryos, indicating that, although these two peptides are blocking receptor sites on the cells to two important basement membrane glycoproteins, it is fibronectin, rather than laminin, that influences the adhesion, and possible migration of the cells. Results from the t-test, at $p<0.05$ indicated a significant difference in the same parameter, between a) embryos treated with RGDS, and those exposed to GRGESP, b) embryos treated with anti-laminin antibodies, and those exposed to anti-fibronectin antibodies, and also between c) embryos treated with the peptide GRGESP, and those exposed to anti-fibronectin antibodies, in addition to two other cases already proven significant by the ANOVA.

Overall, it appears that RGDS and anti-fibronectin antibodies have a greater effect on the migration of the mesoderm cells, than GRGESP, YIGSR, or anti-laminin antibodies.

Further analysis at a higher magnification using SEM, detected an alteration in the shape of the mesoderm cells, between various treatments:

a) between RGDS and GRGESP-treated embryos - this is to be expected, since GRGESP is a control peptide. Obviously, the RGDS peptide is having some effect on the shape of the cells,

b) between GRGESP-treated embryos, and those exposed to anti-laminin antibodies -blocking the cells' access to laminin in the basement membrane appears to have an effect on cell shape,

c) between- GRGESP-treated embryos, and those exposed to YIGSR - blocking the cell receptor for laminin appears to have an effect on cell shape,

d) between GRGESP-treated embryos, and those exposed to anti-fibronectin antibodies - the antibody seems to have an effect on the shape of the cells,

e) between embryos treated with anti-laminin antibodies, and those exposed to anti-fibronectin antibodies - although both these antibodies inhibit the attachment of the mesoderm cells to components of the basement membrane, it is likely that the alteration in shape is due to the cells' inability to utilise fibronectin as a substratum on which to migrate.

In vitro studies using the same peptides and antibodies were carried out as a parallel study to the *in vivo* experiments. This allowed a closer examination of the effects of the various treatments on the mesoderm cells. It has previously been shown that when mesoderm cells from stage 4 embryos are cultured on untreated glass coverslips with normal medium, the cells display a fibroblastic morphology (Sanders, 1980). In the same set of experiments, it was also shown that mesoderm cells, when cultured on fibronectin-coated coverslips with normal medium, could change their morphology and grow as epithelial sheets. These two morphologies provided standards with which to compare the appearance of mesoderm cells under different experimental conditions.

Although mesoderm cells will grow on untreated glass coverslips with normal medium, results from this study show that if the peptides RGDS or YIGSR, or anti-fibronectin or anti-laminin antibodies are added to the medium, the cells are unable to spread out from the main mesoderm mass. This seems to indicate that when grown in normal medium, the cells cultured *in vitro* may be capable of producing their own fibronectin, although probably only in small amounts. However, *in vivo* studies to date have not detected any fibronectin synthesis by the mesoderm cells. Since the fibronectin in the serum was removed prior to the experiments, the possibility of the cells using this for attachment and migration, can be ruled out. Another possibility may be that the cells are using the vitronectin present in the serum as a spreading factor (Straus *et al.*, 1989). Like fibronectin, the vitronectin molecule also has an RGD cell-binding site (Ruoslahti & Pierschbacher, 1987). It has been shown *in vitro* that below 3% serum in culture medium cell spreading is characteristic of that on fibronectin, whereas, above 3%, cell spreading is correlated with that on vitronectin (Knox, 1984). Underwood & Bennett (1989), demonstrated that cells cultured on uncoated plastic in serum-containing tissue culture medium, used vitronectin only, as the operative cell-adhesion and spreading molecule. In this particular study, it may be that if the cells are cultured in a medium containing a blocking agent, however, such as RGDS, YIGSR, anti-fibronectin, or anti-laminin antibodies, the cells may be unable to synthesize sufficient fibronectin, or utilise the vitronectin to the same degree, to be able to attach to the glass, and they, therefore, remain in clumps.

If the mesoderm cells are cultured on fibronectin, however, and treated with medium containing the RGDS peptide, they are not completely inhibited from growing, but display a varied morphology of cell types, including rounded and spread cells, in addition to epithelial sheets. This would lead us to believe that the peptide is causing partial inhibition, but does not entirely prevent the cells from spreading. Since the RGDS peptide is not the only cell-binding region present in fibronectin (see Yamada, 1989), it may be that the mesoderm cells can utilise these other regions, but require all of them to be functional for maximum growth and migration. The cells may also be able to use other binding domains eg. collagen, for their attachment to fibronectin.

When mesoderm cells are cultured on laminin-treated coverslips with normal medium, they appear to grow with the same morphology as the mesoderm cells cultured on untreated glass coverslips with normal medium. This would imply either that the laminin substrate is having little effect on the outgrowth of the cells, or that cells attached to laminin may simply share the same morphology as those attached to glass. If, however, the mesoderm cells are, again cultured on a laminin substrate, but exposed to the YIGSR peptide, they do not spread, but remain as a clump, often unattached to the substrate. This provides a contradiction if it is assumed that the first possibility is true, since these results indicate that YIGSR is inhibiting spreading on laminin. A possible reason for this paradox, may be, that, *in vitro*, where only one variable is being manipulated *ie.* cell-binding to laminin, the YIGSR peptide is capable of inhibiting spreading. The *in vivo* injections also indicated that cell attachment to laminin was not significant for their movement. However, *in vivo*, where mesoderm migration involves more than one factor, cell-laminin binding may not be of such great importance, in comparison with other determinants, and therefore, the inhibition of cell-binding to laminin with YIGSR may be masked by other factors.

Mesoderm cells cultured on laminin-treated coverslips, and exposed to the RGDS peptide, were prevented from spreading. This confirms that the RGDS sequence is also present in laminin (Grant *et al.*, 1989), in addition to fibronectin, and may play a role in the binding of these cells to laminin.

Experiments using anti-fibronectin, and anti-laminin antibodies on cells cultured on fibronectin and laminin respectively, appeared to block cell attachment and spreading completely, and had similar, although not identical effects to the peptides RGDS, and YIGSR, on the same substrates.

The peptide GRGESP, was used in experiments where mesoderm cells were cultured on fibronectin and untreated glass, as a control for experiments using RGDS. The morphology of the cells on each substrate was not affected by the peptide. The IgG antibody was also used as a control, for experiments in which the anti-fibronectin, and anti-laminin antibodies were used. Since IgG is a non-specific antibody, it should not have had an effect on the cells cultured on any of the substrates, unlike the more specific anti-fibronectin and anti-laminin antibodies. Observation of the cultures showed this to be true.

The final batch of experiments involved the addition of anti-fibronectin receptor antibodies *ie*. antibodies to integrin, to mesoderm cells cultured both on untreated-glass, and on fibronectin-coated coverslips. It was noted that those cells cultured on glass and treated with the antibody, failed to spread, although they appeared to have attached to the substrate. Those cells cultured on fibronectin, however, and exposed to the antibody, displayed varied morphologies, similar to those displayed by the same cells when treated with RGDS. This result would be expected, since the anti-fibronectin receptor antibody, and the synthetic RGDS peptide, both block the integrin receptor present on the cell (Bronner-Fraser, 1985; Duband *et al*., 1986; Lash *et al*., 1987).

Experiments using anti-fibronectin receptor in medium surrounding endodermal tissue cultured on glass or fibronectin, did not appear to have any effect on the tissue, since it continued to display its normal epithelial morphology. This would indicate that the spreading of endodermal tissue, unlike mesoderm, is not dependent on the RGDS cell-binding sequence present in fibronectin.

It appears from the *in vivo* experiments carried out, that the fibronectin cell-binding tetrapeptide, RGDS, has some effect on the mesoderm cells when it is microinjected into the gastrulating chick embryo. Visual and statistical observation of the results indicates that the mesoderm cells become more spherical in shape, and are less inclined to make contact with the basement membrane underlying the ectoderm. *In vitro* cultures of the mesoderm cells treated with the same peptide also tend to show alterations in the cells' morphology, although not all the cells appear to be affected. It is apparent from these observations, that the mesoderm cells are utilizing the fibronectin present in the basement membrane for their lateral migration away from the primitive streak. However, the *in vitro* studies suggest that this is not their sole method of migration. It is known that there are sites, other than the RGDS site, which are involved in cell attachment to fibronectin *ie*. CS1 and the site synergistic with the RGDS region. Dufour *et al.*, (1988) demonstrated, for neural crest cells that certain combinations of these sites were required for various cellular functions, for example, cell attachment and cell migration. It has also been demonstrated with neural crest cells, that other binding domains, such as those for collagen and heparin, are capable of supporting the migration of the cells via an RGD-insensitive mechanism (Perris *et al.*, 1989). Migration on the heparin-binding fragment is blocked by the addition of exogenous heparin, and suggests that, *in vitro*, cell attachment to fibronectin may be mediated by cell-surface associated heparan sulphate proteoglycans, in addition to the RGDS receptors.

These observations lead us to conclude that the synthetic peptide, when injected into the embryo, does have an effect on the cells, and consequently indicates that these cells are utilizing the fibronectin in the basement membrane on which to migrate. However, since the RGDS domain is present in other molecules, such as collagen (Ruoslahti & Pierschbacher, 1987), the exogenous peptide may also be affecting the attachment of the cells to other components of the basement membrane.

Two conclusions can be drawn: 1) the mesoderm cells may be able to bind to other regions within the fibronectin molecule, and 2) mesoderm cells may be using other components of the basement membrane, in addition to fibronectin, on which to migrate, either via an RGD-sensitive, or insensitive mechanism.

Laminin was also considered to be a possible substrate used by the mesoderm on which to migrate. Again, visual and statistical observation of embryos treated with YIGSR, showed an alteration in morphology compared with control embryos, although not to the extent of those treated with the RGDS peptide. *In vitro* studies demonstrated the inability of the mesoderm cells to grow with their usual morphology when treated with the same peptide. Treatment of these cells cultured on laminin, with the peptide RGDS, also prevented normal growth, indicating a possible function for this sequence in cell attachment to laminin. It has been demonstrated, however, that the RGD sequence in laminin is located close to a globular domain and that this domain may, in the native form of laminin, block access to the RGD site (Timpl *et al.*, 1990). Therefore, adhesion of the mesoderm cells to laminin via the RGD site may only occur in culture.

It has been demonstrated that laminin has the capability to promote the mobility of some cells, by haptotaxis (McCarthy *et al.*, 1983). Although laminin may be involved in the migration of the mesoderm cells, however, *in vitro* studies have revealed that substrates containing different laminin complexes eg. laminin-nidogen, laminin-collagen type IV, vary in their ability to support neural crest cell migration (Perris *et al.*, 1989). This suggests perhaps that the specific configuration of laminin in its complexed form, may be an important determinant in its motility-promoting ability.

We can therefore, conclude that 1) the mesoderm cells may be able to bind to other regions within the laminin molecule, 2) depending on the molecule to which laminin is bound, the complex may be more or less effective in promoting mesoderm migration, and 3) as previously mentioned, laminin and fibronectin may not be the only molecules used as a substrate for the migration of the mesoderm cells.

Although this study has provided some enlightenment towards the methods of migration of these cells, continuing studies are required to achieve a more complete description. In addition to increasing the sample size for *in vivo* experiments, it would also be of value to block cell attachment to vitronectin with antibodies *in vivo*, and to remove it from the serum *in vitro*, since it may be promoting cell attachment. Receptor specificity for fibronectin versus vitronectin can also be determined by altering the stereochemistry of the Arg-Gly-Asp-Ser sequence (Pierschbacher & Ruoslahti, 1987). In addition, it would be useful to allow the embryos to grow for a longer period of time, after the injection of the peptides, since these particular experiments were carried out with a relatively short time duration. The long term effects of the peptides may prove to be more detrimental than they appear at present. In conjunction with this thought, peptides could also be injected at later stages of development.

Previous experiments have injected both anti-fibronectin antibodies (Boucaut *et al.*, 1984a), and synthetic peptides containing the fibronectin cell-binding region into blastula or early gastrula stages of amphibian embryos (Boucaut *et al.*, 1984b). Injection of antibodies just prior to gastrulation prevented the invagination of mesodermal cells, and caused a gross malformation of the developing embryo. At the blastula stage, embryos injected with the peptide displayed severely malformed animal halves, and there were no migrating cells on the basal surface of the ectoderm on the blastocoel roof. These results are more pronounced than those seen in this project, and may indicate, perhaps, that the involvement of fibronectin in amphibian development is of greater significance than during chick development. In contrast, Boucaut and colleagues (1984b) demonstrated that synthetic peptides containing the cell-binding sequence of fibronectin, caused neural crest cells to detach and round up, when cultured *in vitro*, and prevented their forward migration *in vivo*. These results, both *in vitro*, and *in vivo*, appear comparable to results obtained from this project. It is apparent that these peptides and antibodies will perturb the attachment and migration of cells that rely, in part, at least, on fibronectin for their continuing movement. Although the results from Boucaut's work with chick neural crest are not directly comparable with results from this project, since they involve two different stages, it can be said that the spatial and temporal distribution of fibronectin has a positive function in the continuing development of the chick embryo.

To summarize, it seems likely that the during gastrulation in the chick embryo, the mesoderm cells moving laterally away from the primitive streak, use both fibronectin and laminin present in the basement membrane, on which to migrate. It appears, however, that fibronectin is of greater importance at this time, probably, in part, due to the fact that it is present in greater amounts than laminin. Although the RGDS sequence in fibronectin, and the YIGSR sequence in laminin, are apparently important in mediating attachment of the cells to their respective glycoproteins, it is evident that other domains present within fibronectin and laminin may also be responsible for cell-binding. It is interesting to note, that endodermal tissue taken from stage 4 chick embryos will grow on fibronectin, and is not inhibited by anti-fibronectin receptor antibodies. This implies that the endoderm is not dependent on the integrin receptor for its adhesion to fibronectin, but perhaps, that the endoderm cells are utilizing other regions of the fibronectin molecule via different cell-surface receptors for their adhesion. It seems likely that fibronectin and laminin are not the only molecules involved in mesoderm migration, and that the specific combination of all the possible contributing factors so far discussed, are required in order to achieve maximal cell migration, and the continuing development of the embryo.

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